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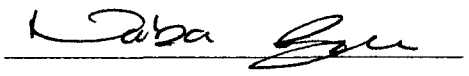

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13. ABSTRACT ***(Maximum 200 Words)***

The identification of the guanine nucleotide exchange factor GEF-H1/KIAA0651 as a substrate of PAK kinases and a mediator of JNK signaling suggested it to be a good candidate for a linker between caspase-mediated Pak2 activation and JNK signaling. As yet, we have no evidence that exchange factor activity is regulated by PAK phosphorylation. Instead, localization of the exchange factor on microtubules may inhibit GEF activity. Studies using microtubule drugs and exchange factor mutants support a model in which RhoA activation through microtubule depolymerization is mediated GEF-H1. In addition, we could show that phosphorylation of GEF-H1/KIAA0651 by Pak recruits 14-3-3 proteins and hypothesize that Pak is involved in regulating protein docking to GEF-H1/KIAA0651.

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TABLE OF CONTENT

INTRODUCTION

The activation of the ubiquitously expressed Pak2 kinase through caspase cleavage might be responsible for the robust activation of the stress-activated MAPK kinase pathway in the apoptotic execution phase. We have identified and characterized a guanine nucleotide exchange factor GEF-H1/KIAA0651 that is a substrate of Pak kinases and a regulator of JNK activity. The localization of the PAK phosphorylation site gave further insight into the mechanism of GEF-HI/KIAA0651 regulation and hints to a phosphorylation-dependent protein-docking mechanism involving 14-3-3 proteins. Intensive studies on the exchange factor activity suggest that exchange activity of GEF-H1/KIAA0651 is modulated through cellular localization. Studies using microtubule-destabilizing drugs support this model and imply that RhoGTPase activation by microtubules is mediated by this exchange factor.

Body

The signaling pathways that are responsible for mediating PAK signaling to the stress-activated MAPK modules are not yet defined, as no PAK substrates have been identified so far that could mediate PAK phosphorylation signals to the SAPK module. That PAK kinases can mediate JNK activation as has been shown independently by several groups (Zhang et al., 1995; Brown et al., 1996; Bagrodia et al., 1995). The PAK homologue STE20 is an activator of an analogous signaling module in yeast involved in the mating response pathway and its signaling mechanism is best understood. STE20 phosphorylates and thereby activates the MAPKKK STE11 (Drogen et al., 2000) although, as has been suggested recently, interactions between several components of the pathway are necessary for proper signaling (Elion, 2000). In mammalian cells, data suggest that PAK might not necessarily mediate signals to MAPK modules as a MAPKKK kinase. It has been demonstrated for the mitogen-activated protein kinase pathway that Raf kinase (King et al., 1998) as well as MEK (Frost et al., 1997) can be phosphorylated by PAK. In these cases, phosphorylation alone is not a sufficient but necessary event for a proper MAPK signaling response.

Our initial observations in breast cancer cells [annual report 2000] suggested that PAK2 activation during apoptosis, which is mediated by caspase cleavage (Rudel and Bokoch, 1997), is responsible for the activation of JNK. Inhibitors of caspases strongly reduced or abolished activation of JNK during apoptosis, indicating that a caspase-cleavage event was necessary. Data from studies in T lymphocytes using a dominant-negative PAK construct indicated that PAK is required for JNK activation during apoptosis (Rudel et al., 1998).

We therefore initiated a biochemical screen combined with mass spectrometric fingerprinting to identify new PAK substrates [annual report 2000]. Using this approach we could unambiguously identify the guanine nucleotide exchange factor GEF-H1/KIAA0651 as a new substrate of PAK kinases. GEF-H1 was described as a microtubule-localized GEF for Rho and Rac (Ren et al., 1998) whereas KIAA0651 was identified by sequencing of large cDNA fragments from human brain (Ishikawa et al., 1998). As we found out by sequencing and database searching, KIAA0651 and GEF-H1 originate from the same genomic locus by alternative splicing [annual report 2001]. During 2001 we identified a single phosphorylation site (serine 885) of PAK in

GEF-H1. Data from Cos-1 cell transfection studies indicated that GEF-H1 could potentially regulate JNK activation but a connection to PAK phosphorylation remained unclear. Guanine nucleotide exchange studies indicated that GEF-H1/KIAA0651 was a good activator of RhoA but did not exchange nucleotide on Rac as initially reported by Ren et al. (1998). Localization of GEF-H1 on microtubules was verified in transiently transfected HeLa cells using indirect immunofluorescence techniques in collaboration with Mira Krendel, research associate in Dr. Bokoch's laboratory.

During the final year of this postdoctoral fellowship, Dr. Diebold and I tried to get further insight into the cellular function of the guanine nucleotide exchange factor GEF-H1 that I found to be a substrate of p21-activated protein kinase PAK [technical objective 3]. As described in my annual report of 2001, we were able to map a single site in GEF-H1 that was phosphorylated by PAK. Mutation of this residue to alanine totally abolished phosphorylation as shown by *in vitro* kinase assays.

Using the protein motif search program ScanSite (Yaffe et al., 2001) we identified a 14-3-3 binding motif which is centered around the identified PAK phosphorylation site (Fig1). 14-3-3 proteins belong to a family of basic proteins, which have been shown to bind to and regulate the activity of a variety of other proteins (Fu et al., 2000). In general, these proteins only interact, when the binding motif in the respective interacting protein is phosphorylated (typically at the centrally located serine residue). Well-known examples of proteins interacting with 14-3-3 are the proapoptotic protein Bad and the protein kinase Raf. Phosphorylation of Bad by Akt or other kinases (see introduction of Masters et al., 2001) enables its interaction with 14-3-3 proteins and subsequent sequestration of Bad in the cytoplasm away from its interacting partner Bcl-2; the proapoptotic function is thereby inhibited. On the other hand it is well established that the protein kinase Raf interacts with 14-3-3 in order to become fully activated.

We hypothesized that PAK phosphorylation might regulate the interaction of GEF-H11 with 14-3-3. To test this, we used recombinant GST-GEF-H1[1] (aa 571-985) and phosphorylated this protein with GST-PAK. The glutathione agarose-bound GST-GEF-H1 (aa 571-985) was

1 Note, that the sequence of GEF-H1 was corrected in length to 985 amino acids. This difference of one amino acid is due to the absence of one amino acid (alanine 194, IDEAEVIYSE, see XP_044171) in the originally received GEF-H1 cDNA from Ren et al (1998). Interestingly, this amino acid is located right at a splice border.

incubated for one hour with total brain cell lysate, washed, and analyzed for 14-3-3 binding by western blotting. As seen in Fig.1, 14-3-3 specifically bound to phosphorylated GST-GEF-H1 (aa 571-985) and not to unphosphorylated GEF-H1 or GST as a control. In addition, we could show that phosphorylation of serine 885 is crucial for the interaction with 14-3-3. A GST-GEF-H1 (aa 571-985) containing a mutation from serine to alanine at position 885 did not bind 14-3-3 even when pre-incubated with GST-PAK/ATP under kinase assay conditions (Fig.1, lane 5).

In co-transfection experiments we could show that ectopically expressed GEF-H1 interacts with 14-3-3 only when expressed together with an active but not inactive form of PAK (data not shown). Together, these data clearly demonstrate that 14-3-3 interacts with GST-GEF-H1 in a phosphorylation-dependent manner, confirming the prediction by the ScanSite program.

Interestingly, the motif search program predicted the presence of an SH3-binding domain overlapping with the 14-3-3 binding site (Fig.1, upper panel). The overlap of the binding motifs immediately suggested an attractive model of how Pak could influence a protein-protein interaction via phosphorylation-dependent docking of 14-3-3.

According to ScanSite, this proline-rich sequence (Fig.1, upper panel) is predicted to bind to the SH3 domain-containing protein amphiphysin. Amphiphysin is a protein involved in vesicular transport processes and has been shown to interact with components of the endocytic transport machinery (Wigge and McMahon, 1998). We therefore tested whether amphiphysin is able to interact with Gef-H1. Immobilized GST-GEF-H1 (aa 571-985) was incubated with total Jurkat cell lysates, washed and analyzed by western blotting for the presence of amphiphysin. However, under our assay conditions we were not able to detect any amphiphysin in the pulldown reactions. Conversely, GST-Amphiphysin (generous gift from S.Schmid, TSRI) was used to attempt to pull down endogenous GEF-H1 from Jurkat lysates or ectopically expressed GEF-H1 from Cos-1 cell lysates. Again, we were unable to detect any interaction between these two proteins.

From binding studies using the SH3 adaptor protein Nck or Pix (PAK-interacting exchange factor) and the known binding regions of the protein kinase PAK1, we know that we can detect SH3/PXXP type of interactions using our specific binding buffer and assay conditions (data not shown). Thus, we consider it likely that an unknown SH3 domain containing protein is binding to this region and therefore might need to employ screening-based identification methods

to identify potential binding partners (e.g. yeast two-hybrid, biochemical screening). These procedures have been initiated by Drs. Diebold, Krendel and Jeanclos in the Bokoch laboratory.

As outlined in my last report, we were able to measure guanine nucleotide exchange factor activity toward the small GTPase RhoA, but not Rac1. More recently, we have determined that Cdc42 is not exchanged by GEF-H1 either (Fig.2, upper panel). Exchange factor specificity was determined using epitope-tagged GEF-H1 protein (HA-GEF-H1) produced in Cos-1 cells. Using the promiscuous exchange factor, Dbl, ensured that all recombinant GTPases are functional as substrates in the assay. The exchange of RhoA by GEF-H1 was time-dependent and no significant background was observed (Fig.2, lower panel).

We attempted to measure the Rho activation status using a recently developed binding and precipitation protocol (Ren and Schwartz, 2000). This method is based on the principle that active RhoGTPases can be specifically affinity-purified on immoblilized GST-RBD proteins and subsequently detected by western blotting. We were unable to detect any activation of endogenous RhoA in Cos-1 or HeLa cells transfected with GEF-H1/KIAA0651 using this pulldown method. However, when GEF-H1 was co-expressed with epitope-tagged myc-RhoA in Cos-1 cells activation of RhoA was observed (Fig.3, lane 3).

Treisman and colleagues (Hill et al., 1995) have described a reporter gene system, which is responsive to the activity status of the small GTPases Rho, Rac, and Cdc42. In this system the reporter gene is under control of a serum response element (SRE). The serum response element is a complex DNA binding motif and under control of a variety of signaling pathways (Treisman, 1996). As Treisman and colleagues have shown, activated forms of RhoA, Rac1 and Cdc42, but not the inactive forms, are capable of stimulating reporter gene expression demonstrating that the activity status of RhoGTPases can trigger SRE activation. We transfected GEF-H1 constructs together with an SRE-driven luciferase gene into Cos-1 cells and measured reporter gene expression in comparison to control transfected cells. GEF-H1$^{1-985}$, GEF-H1$^{1-895}$, GEF-H1$^{1-572}$, and KIAA0651 all showed significant activation of the reporter gene in comparison to vector-transfected cells (Fig.4). However, activation was substantially higher when cells were co-transfected with the guanine-nucleotide exchange factor Dbl or activated RhoGTPases (RhoAQ63L, Rac1Q61L, Cdc42Q61L).

To analyze whether GEF-H1/KIAA0651-mediated SRE reporter gene activation is due to activation of RhoGTPases, we used the co-expression of GTPase binding domains isolated from their respective effectors. The GTPase-binding domain of the Rho effector Rhotekin (also called RBD) binds specifically to RhoA, whereas the p21-binding domain of PAK (PBD) binds Rac and Cdc42. Overexpression of the respective domains neutralizes activated GTPases through selective sequestration. Interestingly, co-transfection of RBD but not PBD abolished reporter gene expression (Fig.5). This consistent with our *in vitro* exchange factor data (see Fig.2 & annual report 2001), demonstrating that only RhoA, and not Rac1 and Cdc42, is a substrate of the GEF-H1 exchange factor. We mutated an essential residue in the catalytic Dbl homology domain to assess the role of exchange activity in SRE activation. Mutating tyrosine 393 to alanine in GEF-H1/KIAA0651 abolished reporter gene activation completely (Fig.5) indicating that a functional Dbl homology domain is necessary to activate the SRE reporter gene.

As confirmed by several independent measurements the above-employed GEF constructs differ in their potential to mediate SRE activation (Fig. 4). The full-length constructs GEF-H1$^{1\text{-}985}$, and GEF-H1$^{1\text{-}895}$ consistently yield 2-3 times less reporter gene activity than GEF-H1$^{1\text{-}572}$ or KIAA0651. As we could show by western blotting, this is not due to differences in expression in Cos-1 cells. Thus, C- and N-terminal regions outside the catalytic Dbl homology domain (note that KIAA0651 differs from GEF-H1 only in the amino-terminus; see annual report 2001) may be important in regulating exchange activity.

The above SRE reporter gene measurements suggest that exchange activities of the GEF-H1/KIAA0651 constructs are different. To analyze this, we immunoprecipitated Hemagglutinin-tagged exchange factor variants (Gef-H1$^{1\text{-}985}$, GEF-H1$^{1\text{-}895}$, GEFH1$^{1\text{-}572}$, KIAA0651) from Cos-1 cell lysates and compared *in vitro* exchange activities (Fig.6). However, we could not detect any difference in their abilities to exchange recombinant RhoA. Additionally, the same GEF-H1/KIAA0651 constructs did not differ in their potential to activate co-expressed myc-tagged RhoA as analyzed using the RBD pulldown assay (Fig.3, lanes 3-6), although we have to treat these results with caution since the overexpression of RhoA leads to a significant autoactivation (Fig.3, lane 1).

In collaboration with Mira Krendel, research associate in the laboratory of Dr. Bokoch, we investigated the cellular localization of the different GEF-H1/KIAA0651 constructs in HeLa

cells. Hemagglutinin- or eGFP-tagged GEF constructs were transfected into HeLa cells and visualized by indirect immunofluorescence or direct fluorescence techniques, respectively. GEF-H1$^{1-985}$ and Gef-H1$^{1-895}$ clearly localized to a fibrous network, which we determined to be the microtubule network using co-staining with anti-tubulin antibodies (Fig.7). Overexpression often led to bundling of microtubules (see GEF-H1 transfected HeLa cells, Fig.6B) and is often seen for microtubule-associated proteins. GEF-H1$^{1-572}$ and KIAA0651 are diffusely localized in the cell and seem not to be associated with cytoskeletal structures. We conclude that determinants in the C- and N-terminus are required for the localization of GEF-H1 on the microtubules.

As discussed in the previous annual report 2001, we have evidence that KIAA0651 exists in two isoforms, one of which we repeatedly failed to isolate by RT-PCR. The missing longer form (KIAA0651-2) contains an amino-terminal zinc finger motif, also present in GEF-H1, which might be important for localization. This motif is missing in KIAA0651-1, the isoform we originally received from the Kazusa DNA Research Institute (Ishikawa et al., 1998). Indeed, mutating the zinc finger in GEF-H1$^{1-985}$ (cystein 53 to arginine) is sufficient to abolish localization to microtubules (Krendel, personal communication).

Most notably, the above data leave the impression that localization of GEF-H1 and its activity status may be intimately connected. Microtubule-localizing GEF proteins are less active than cytoplasmically localized versions, suggesting that microtubule binding inhibits exchange activity. Reports from several groups have indicated that the microtubule polymerization status regulates RhoGTPase activation (Wittmann and Waterman-Storer, 2001). We therefore tested whether microtubule drugs influence SRE activation in a RhoGTPase-dependent manner.

As shown in Fig.8 the two microtubule-depolymerizing drugs nocodazole and colchicine mediate substantial activation of luciferase expression, whereas the microtubule-stabilizing drug taxol has no effect. Nocodazole–induced SRE activation was inhibited by the Rho binding domain RBD but not the PBD fragment (Fig.8). This strongly suggested that an exchange factor with a similar inhibitory profile like GEF-H1 (see Fig.5) might be activated through microtubule depolymerization to stimulate the RhoA GTPase.

To obtain additional evidence for an involvement of GEF-H1 in microtubule-dependent RhoA activation, we tested whether fragments or mutationally inactivated versions of GEF-H1 act inhibitory in the reporter gene assay. Cos-1 cells were transfected with various GEF-

constructs (comprising amino acids 1-236, 237-570, 571-985 or Y393A mutants of GEF-H1$^{1-985}$, GEF-H1$^{1-572}$ and KIAA0651) and stimulated with nocodazole. This screening showed that only the DH mutants of GEF/KIAA0651 (GEF-H1$^{1-985}$-dh, GEF-H1$^{1-572}$-dh, KIAA0651-dh) have a moderate potential to block nocodazole-induced SRE activation. The inhibition of nocodazole- and colchicine-induced SRE activation by the DH-mutant of GEF-H1$^{1-985}$ as shown in Fig.10 is concentration-dependent. These data suggest that the inactivated GEF-H1 molecules might interfere with nocodazole-induced activation of Rho, probably by blocking the activity of a functionally equivalent, if not identical exchange factor, in Cos-1 cells.

It was obvious to analyze whether exchange activity can be regulated by Pak phosphorylation at serine 885. In order to address this question we co-transfected GEF-H1 with active/ inactive variants of Pak1 in Cos-1 cells and measured activation of the SRE reporter gene. Active Pak1 (Pak1, 83,86L T423E) strongly suppressed SRE-activation, whereas inactive Pak1 (Pak1, 83,86L K299R) did not have an influence. However, this effect was not dependent on GEF-H1 phosphorylation, since inhibition was also observed in the presence of GEF-H1, S885A or GEF-H1 (1-572) in which the total C-terminus was deleted. Furthermore, Pak-phosphorylated GEF-H1 was indistinguishable from non-phosphorylated protein in *in vitro* exchange assays, again suggesting that Pak phosphorylation might not regulate exchange activity.

In this reporting period we have been able to characterize two functional aspects of the recently identified PAK substrate GEF-H1/KIAA0651. The role of phosphorylation of GEF-H1 on serine 885 by the p21-activated protein kinase Pak and the guanine nucleotide exchange activity and its proposed regulation by microtubule localization.

As suggested by our results, Pak phosphorylation on serine 885 creates a docking site for 14-3-3 proteins, a family of small basic proteins that regulate a variety of activities of diverse cellular proteins through phosphorylation-dependent protein-protein interaction. Pak phosphorylation neither modulates exchange activity nor localization of GEF-H1 on microtubules (Mira Krendel, pers. communication) but is hypothesized to regulate interaction with a putative SH3 domain-containing protein that remains to be identified.

The direct and indirect measurements of exchange activity suggest that GEF-H1 specifically activates the GTPase RhoA and not Rac1 or Cdc42. This is in clear contrast to

recently published data indicating that GEF-H1 is active toward Rac1, too (Ren et al., 1999, Gao et al., 2001). A comparison of GEF-H1 variants that differ in their cellular localization are differentially active in SRE reporter gene assays. Cytoplasmically localized variants are considerably more active that microtubule-binding variants; indeed, it is sufficient to alter the localization by a single amino acid exchange in the amino terminal zinc finger domain (C53R) to destroy microtubule localization and increase its in SRE activation potential in parallel. These data strongly suggest that exchange activity might be regulated through microtubule localization. Support for this hypothesis comes from the use of microtubule-depolymerizing drugs that increase SRE activation possibly through a similar mechanism.

KEY RESEARCH ACCOMPLISHMENTS

1. **Reporting period January 1999 to January 2000**
    - Sensitivity characteristics of various breast cancer cells towards different apoptotic stimuli
    - Correlation between Pak2 cleavage and JNK activation in Fas-induced apoptosis
    - Inhibition of caspase-cleaved and activated Pak2 by a Pak1-derived autoinhibitory peptide
    - Establishment of a cytochrome c injection model to study Pak2 effects during apoptosis
    - Identification of KIAA0651/GEF-H1 as a binding partner and substrate of p21-activated kinase, part I

2. **Reporting period February 2000 to January 2001**
    - Identification of GEF-H1/KIAA0651 as a substrate of PAK kinases, part II
    - Correction of sequencing errors in GEF-H1
    - Assignment of a PAK binding region in GEF-H1/KIAA0651
    - Identification of the phosphorylation site in GEF-H1/KIAA0651
    - Preparation of GEF-H1/KIAA0651 antiserum
    - Measurement of exchange factor activity and determination of specificity, part I
    - Activation of JNK by the DH-PH exchange factor domain of GEF-H1/KIAA0651

3. **Reporting period February 2001 to January 2002**
    - Binding of 14-3-3 proteins to phosphorylated GEF-H1
    - Determination of exchange factor specificity using *in vitro* exchange assays, part II
    - Modulation of GEF-H1 activity through microtubule localization
    - Evidence for an involvement of GEF-H1 in microtubule-mediated signaling to RhoGTPases

REPORTABLE OUTCOMES

- King, C.C., **Zenke, F.T.**, Dawson, P.E., Dutil, E.M., Newton, A.C., Hemmings, B.A., Knaus, U.G, Bokoch, GM. Sphingosine–stimulated 3-phosphoinositide-dependent kinase-1 (PDK1) phosphorylates p21-activated kinase (Pak1). J.Biol.Chem. 2000 Jun 16;275(24):18108-13
- **Zenke, F.T.** and Bokoch, GM. The role of caspase-mediated Pak2 activation. Keystone Symposium in Breckenridge, Colorado on programmed cell death, 4/6/99 to 4/11/99
- **Zenke, F.T.**, King, C.C. and Bokoch, GM. Regulation of Pak activity by autoinhibition and phosphorylation. San Diego Cell Biology Meeting, 5/20/99.
- King, C.C., Gardiner, E.M., **Zenke, F.T.**, Bohl, B.P., Newton, A.C., Hemmings B.A., Bokoch, GM. p21-activated kinase (PAK1) is phosphorylated and activated by 3-phosphoinositide-dependent kinase-1 (PDK1). J.Biol.Chem. 2000 Dec 29;275(52):41201-9
- **Zenke, F.T.** Function of PAK Kinase activity in Apoptosis. Poster presentation DOD BCRCP Meeting, Atlanta, June 8-11, 2000
- Krendel, M., **Zenke, F.T.** and Bokoch G.M. Nucleotide exchange factor GEF-H1 mediates cross-talk between microtubules and the actin cytoskeleton. Submitted to Nature Cell Biology
- Krendel, M., **Zenke, F.T.**, Ren, Y. and Bokoch, G.M. Cross-talk between Microtubules and the Actin Cytoskeleton Mediated by GEF-H1 Nucleotide Exchange Factor. Poster presentation ASCB Meeting, Washington December 8-12, 2001
- **Zenke, F.T.**, Krendel, M., King, C.C., DerMardirossian, C., Diebold, B. A., Bohl, B.P. and Bokoch, G.M. Identification of GEF-H1, a microtubule-localized exchange factor, as a substrate of PAK kinases. In preparation.
- Appointment as Senior Cell Biologist in the Department of Oncology, Preclinical R&D, Biomedical Research, Merck KGaA, Frankfurter Straße 250, 64293 Darmstadt, Germany since October 2001

CONCLUSIONS

The guanine nucleotide exchange factor GEF-H1 was characterized regarding the function of the identified PAK phosphorylation site, serine 885, and exchange factor activity. We found that 14-3-3 proteins bind to the Pak-phosphorylated but not to the unphosphorylated GEF-H1/KIAA0651 carboxy-terminal region providing a means to regulate protein-protein interaction in a Pak-dependent manner. However, further work has to identify a role of this phosphorylation-dependent protein docking mechanism.

Gef-H1/KIAA0651 exhibited exchange factor activity toward RhoA, but not Rac1 or Cdc42. Immunolocalization studies showed that GEF-H1 but not KIAA0651 localized to the microtubule network. Interestingly, we observed a correlation between the localization and SRE reporter gene activation in that microtubule-binding variants are less active than non-binding GEF proteins. We do not observe these differences in exchange activities using *in vitro* exchange assays or Rho precipitation studies maybe because of a loss of association with microtubules or overexpression conditions, respectively. Microtubule-depolymerizing drugs appear to activate the RhoA signaling pathway which might be mediated by GEF-H1 or a functionally related exchange factor.

We did not find any evidence that Pak phosphorylation modulates exchange activity or change the localization of GEF-H1 and suggest that Pak regulates a distinct aspect of GEF-H1 function.

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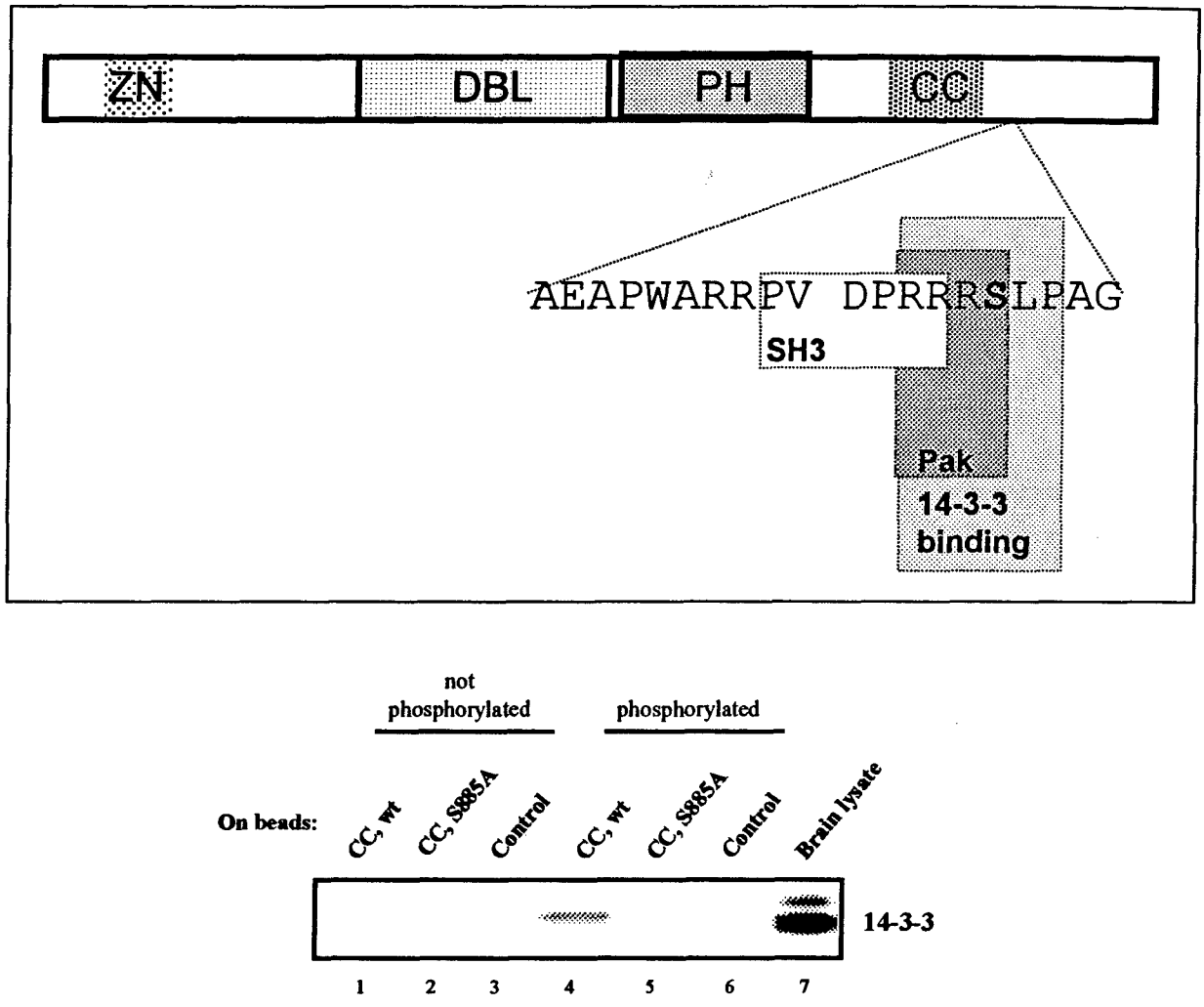
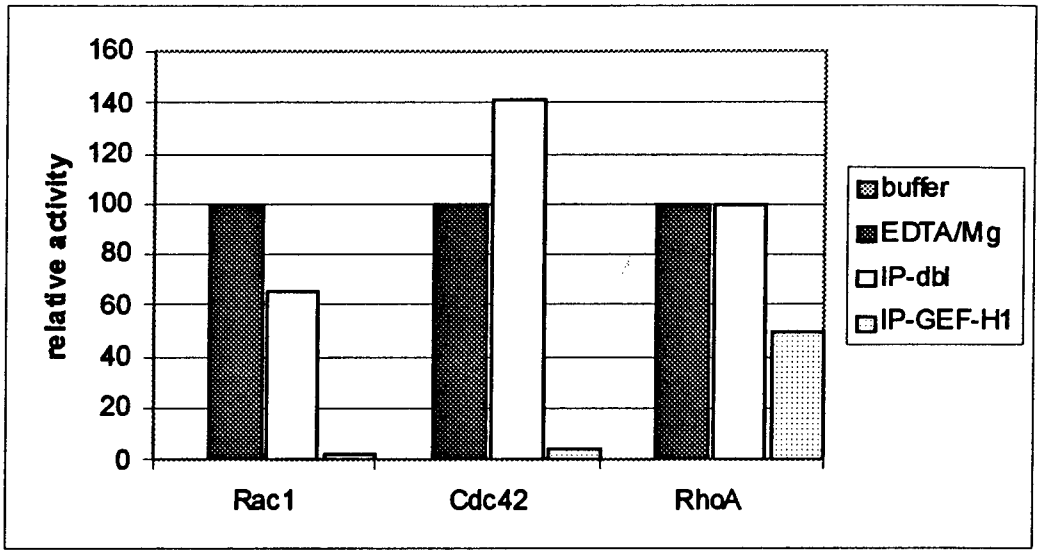


Fig.1: Upper panel: Sequence motifs in the vicinity of the PAK phosphorylation site. A schematic representation of GEF-H1 including the recognizable domain motifs (ZN= zinc finger; DBL = exchange factor domain; PH = pleckstrin homology domain; CC = coiled coil domain) is shown to clear up the position of the PAK phosphorylation site (Serine 885 in bold) and the predicted SH3 and 14-3-3 binding domains. The latter two domains were predicted by the motif search software Scansite (Yaffe et al., 2001). **Lower panel: Phosphorylation-dependent interaction between GEF-H1 and 14-3-3.** GST-fusion proteins containing amino acids 572-985 of GEF-H1 and the corresponding serine 885 to alanine mutant (CC, S885A) were immobilized on glutathione agarose beads. Glutathione S-transferase bound to beads was used as a control. Equal amounts of GST proteins were used in each binding reaction (not shown). The immobilized proteins were either left untreated (lanes 1-3) or phosphorylated for 30 min in the presence of GST-PAK and 0.5 mM ATP. Samples were washed and incubated with equal amounts of bovine brain lysate for about 1 hour at 4°C. Binding reactions were washed, separated by SDS-polyacrylamide gelelectrophoresis and processed for western blotting using a polyclonal anti-14-3-3 antiserum. In lane 7 bovine brain cytosol was loaded.

Specificity for RhoGTPases:



Time kinetic:

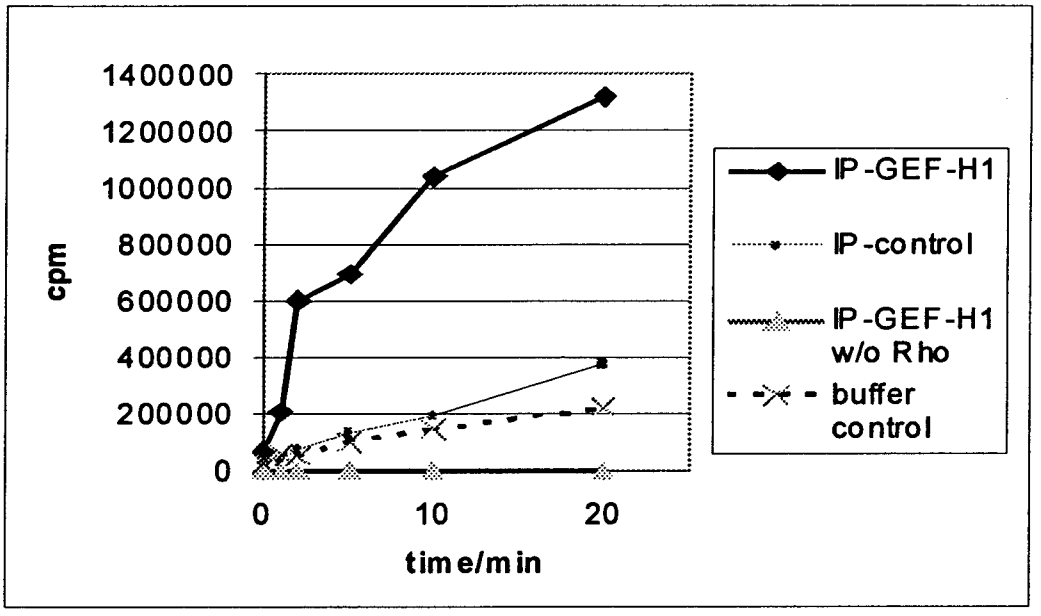


Fig.2: *In vitro* exchange factor assays. Hemagglutinin-tagged GEF-H1 was immunoprecipitated from Cos-1 cell lysates and used for *in vitro* exchange factor assays. A protocol using immunoprecipitates was followed with minor modifications (Downward et al., 1995). **Upper panel:** Recombinant GDP-loaded RhoA, Rac1 and Cdc42 were incubated for 20 minutes with GEF-H1 immunoprecipitates in the presence of ^{35}S-labelled GTPγS. As controls the GTPases were loaded in the presence of buffer (normalized to zero) and EDTA/Mg, which should result in complete non-enzymatic exchange (set to 100%). Additionally, we used immunoprecipitated dbl exchange factor, which acts on all three GTPases. **Lower panel:** 35S-GTPγS exchange was followed over a period of 20 minutes using immunoprecipitated GEF-H1 in the presence (IP-GEF-H1) and absence (IP-GEF-H1 w/o Rho) of RhoA. Anti HA-immunoprecipitate from Cos-1 control lysates (IP-control) and buffer (buffer control) were used as further controls.

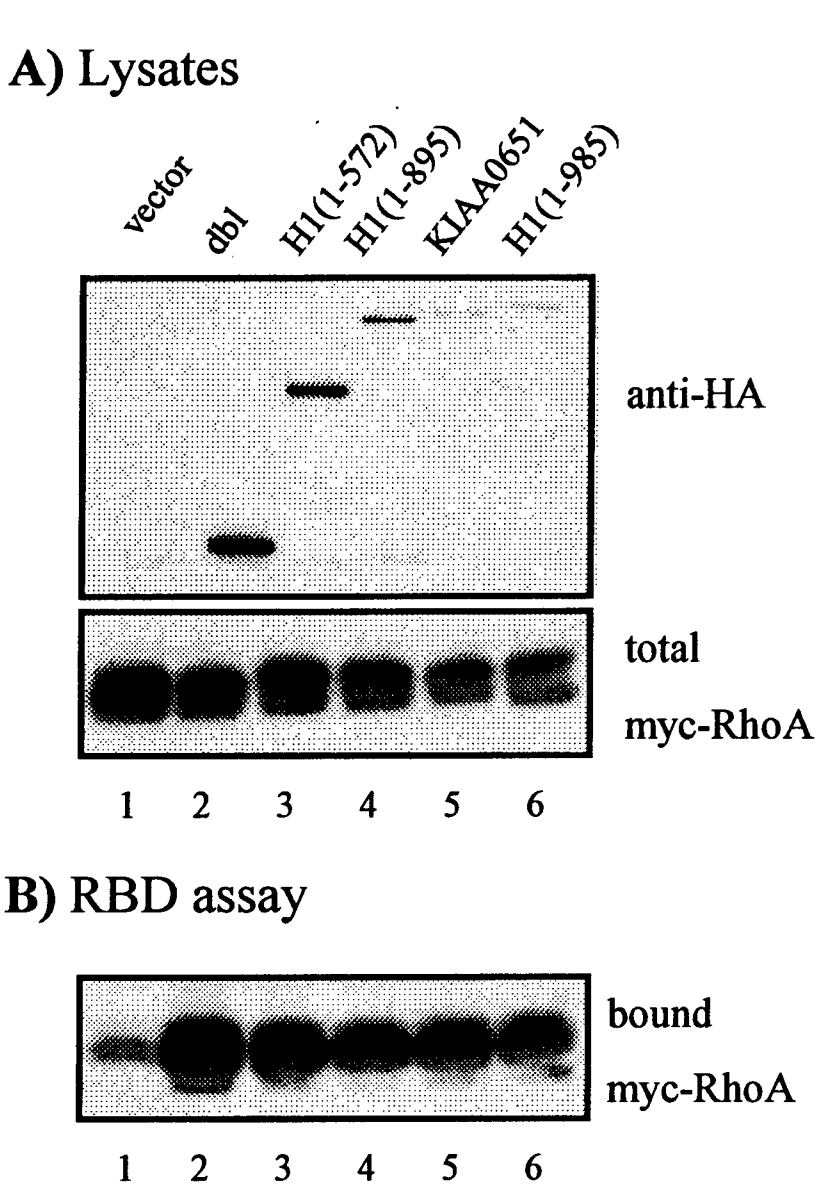


Fig.3: RBD pulldown assay. Cos-1 cells were co-transfected with the indicated GEF-H1/KIAA0651 constructs, dbl or vector control (pCMV5HA3) and myc-RhoA expression vector. 48 hours post-transfection cells were lysed and a Rho binding assay RBD was performed. **A)** Expression of hemagglutinin-tagged GEF-H1/KIAA0651 constructs and myc-RhoA in total Cos-1 cell lysates. Monoclonal anti-HA and anti-myc antibodies were used for detection. **B)** Detection of myc-RhoA in the GST-RBD pulldown fraction. Note the significant level of RhoA protein in control vector transfected cells (lane1). We could verify in reporter gene assays that overexpression of wildtype RhoA alone activates the serum response element (data not shown) suggesting autoactivation upon overexpression.

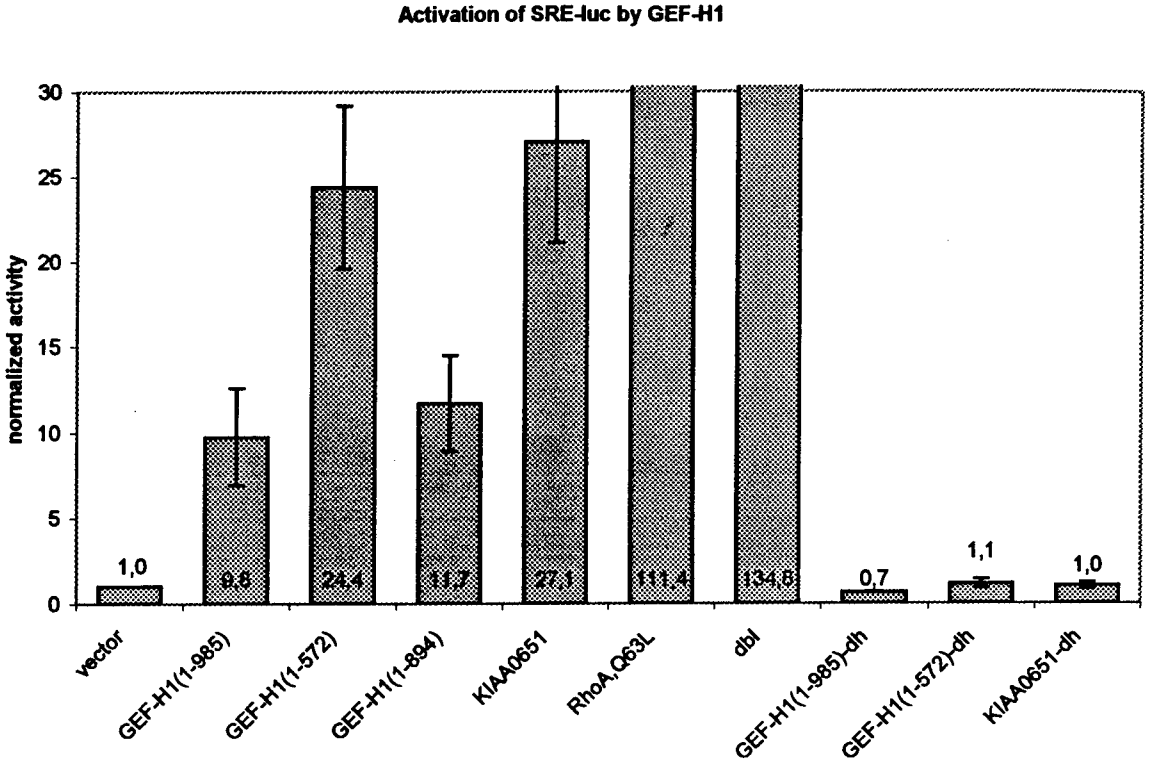


Fig. 4: Influence of GEF-H1/KIAA0651 constructs on SRE-luciferase expression. Cos-1 cells plated in six-well dishes were transfected with 0.5 µg of GEF-H1/KIAA0651 constructs (HA-epitope tagged, inserted into pCMV5) and 0.75 µg of a 2:1 mixture of SRE-luc/pCMV5-lacZ. As controls we used pCMV5HA3 (vector control), pCMV5-dbl (oncogenic form of the RhoGTPase exchange factor dbl) and pRK5-RhoAQ63L (mutationally activated mutant of RhoA). Cells were transfected using Lipofectamine (Gibco, BRL) according to the manufacturers suggestions except that the serum concentration was kept at 0.5% throughout the transfection period. Cells were processed 48 hrs post transfection for luciferase and ß-galactosidase measurements using the Luciferase assay kit (Promega) and the Galactolight kit (Tropix). Shown are the normalized activities (luciferase/lacZ, vector control was set at 1). The mean values are shown in the columns with standard deviation. The data represent a summary of at least three independent experiments.

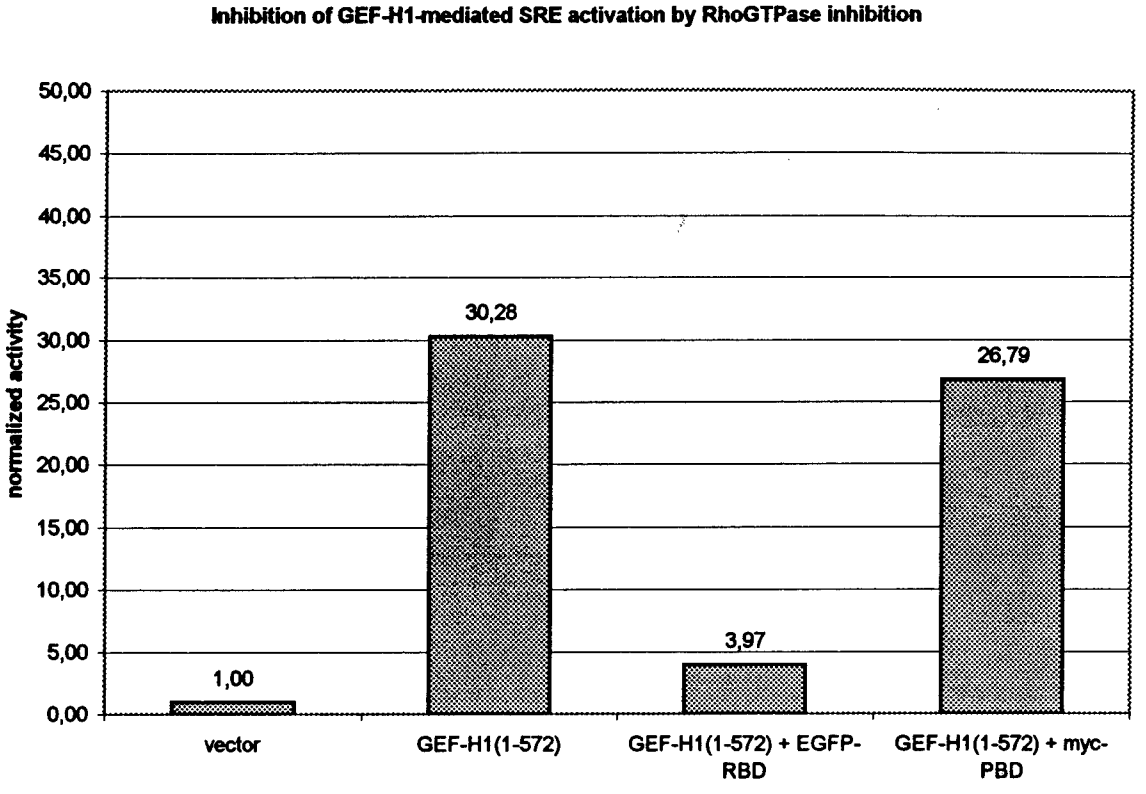


Fig. 5: The Rho binding domain of Rhotekin inhibits GEF-H1-mediated SRE activation. Cos-1 cells were transfected with 0.5 µg GEF-H1 (1-572) and 0.5 µg of dominant negative constructs, eGFP-RBD fusion or myc-tagged PBD (Rac/Cdc42 binding domain of Pak1), together with 0.75 µg of a 2:1 mixture of SRE-luc/pCMV5-lacZ. 48 hrs post transfection luciferase/ lacZ activity was determined. A representative experiment is shown.

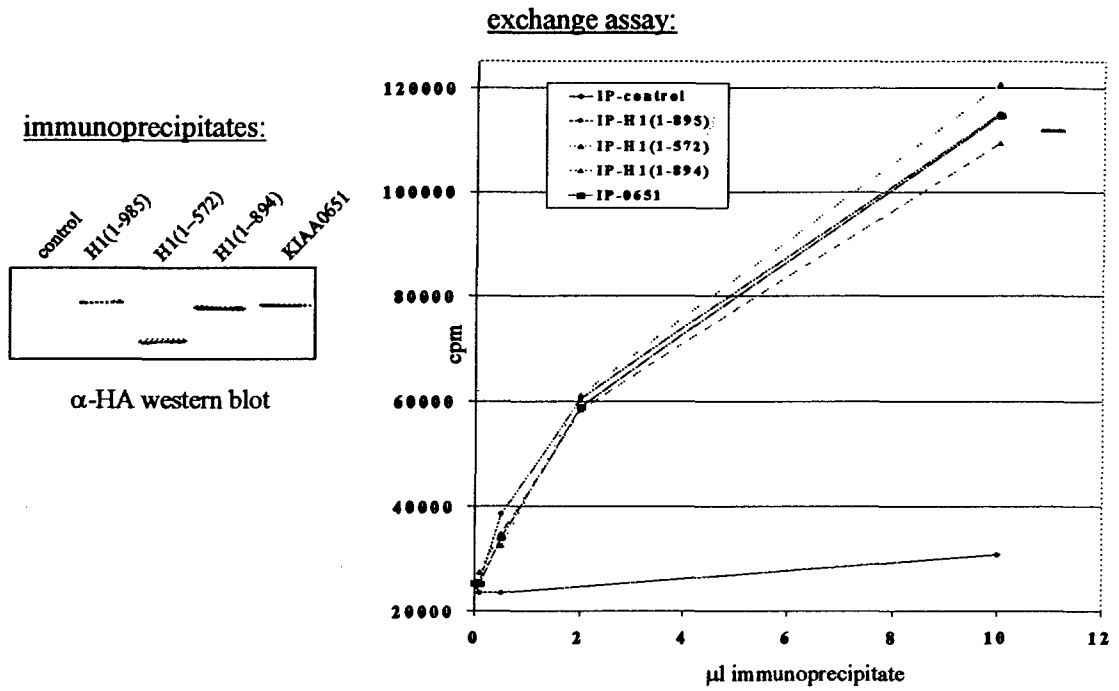


Fig.6: *In vitro* exchange activities of microtubule-binding and non-binding variants. Immunoprecipitated GEF-H1/KIAA0651 variants (amino acid positions for GEF-H1 proteins are indicated) from Cos-1 cells were incubated with recombinant RhoA and 35S-GTP□S for 20 minutes. The loading of the radiolabelled nucleotide is plotted against increasing amounts of immunoprecipitate. By this, we ensured to use exchange factor under non-saturating conditions. Maximum exchange as determined by EDTA-/Mg-extraction is shown as a small horizontal bar in the graph. Western blotting verified (left panel) that the amount of the different GEF-variants used in the exchange assay is similar. Note, that all GEF constructs did not exhibit any activity toward the GTPase Rac1 (data not shown).

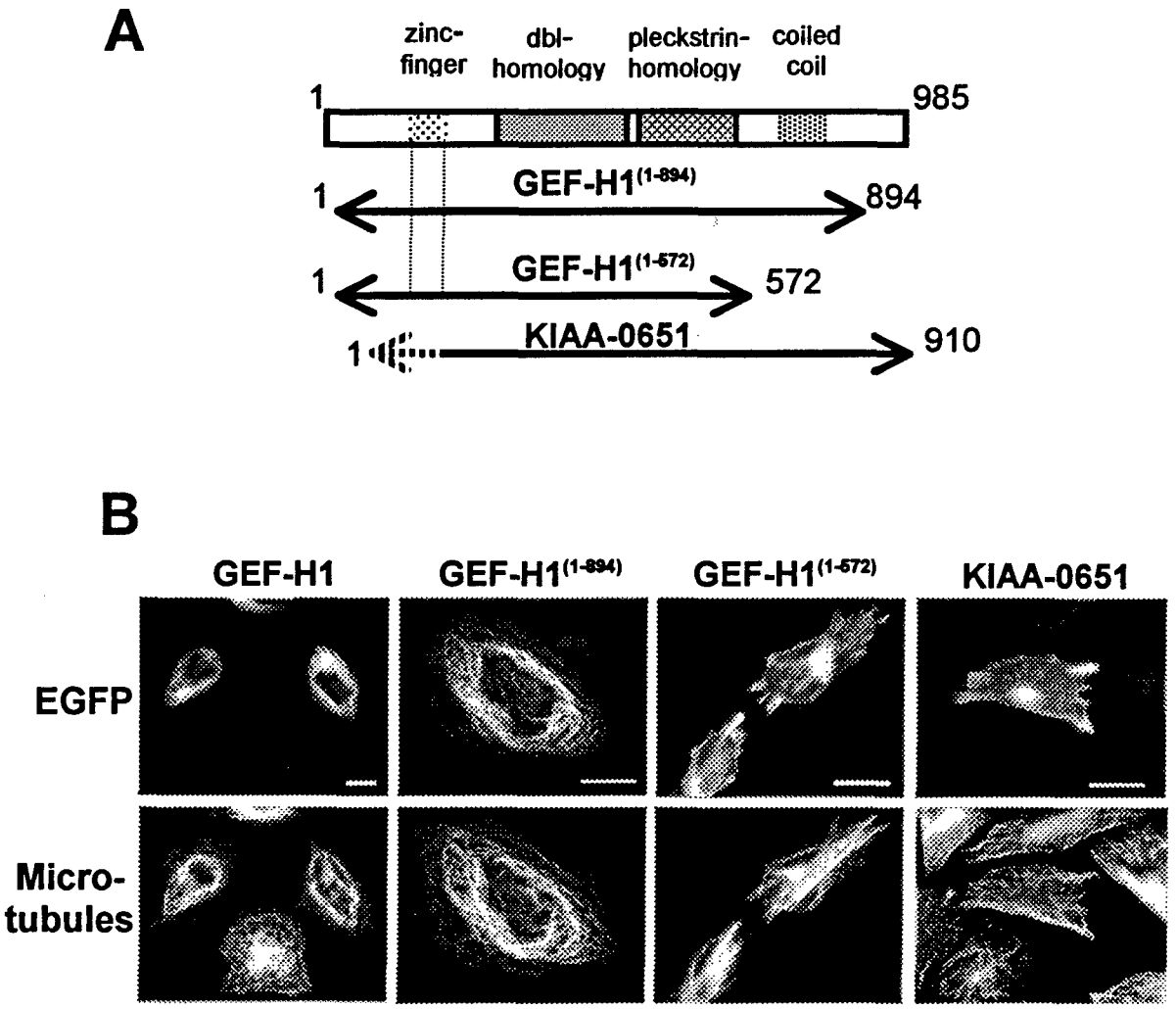


Fig.7: Panel A. GEF-H1/KIAA0651 constructs. A schematic drawing showing the domain structure of the full length GEF-H1 (amino acids 1-985) and various expression constructs used in this study with the positions of the first and the last amino acid indicated. The protein encoded by the KIAA-0651 cDNA clone is identical to GEF-H1 along most of its length (solid line) but differs from GEF-H1 in the N-terminus (dashed line). **Panel B. Intracellular localization of GEF-H1/KIAA0651 constructs.** HeLa cells transfected with EGFP-tagged GEF-H1 constructs were fixed and stained with anti-tubulin antibody. Full-length GEF-H1$^{1-985}$ and GEF-H1$^{1-894}$ co-localized with microtubules. GEF-H1$^{1-572}$ and KIAA0651 exhibited mostly diffuse cytoplasmic localization with some enrichment at the tips of the projections of the cell edge. Scale bars – 20 μm.

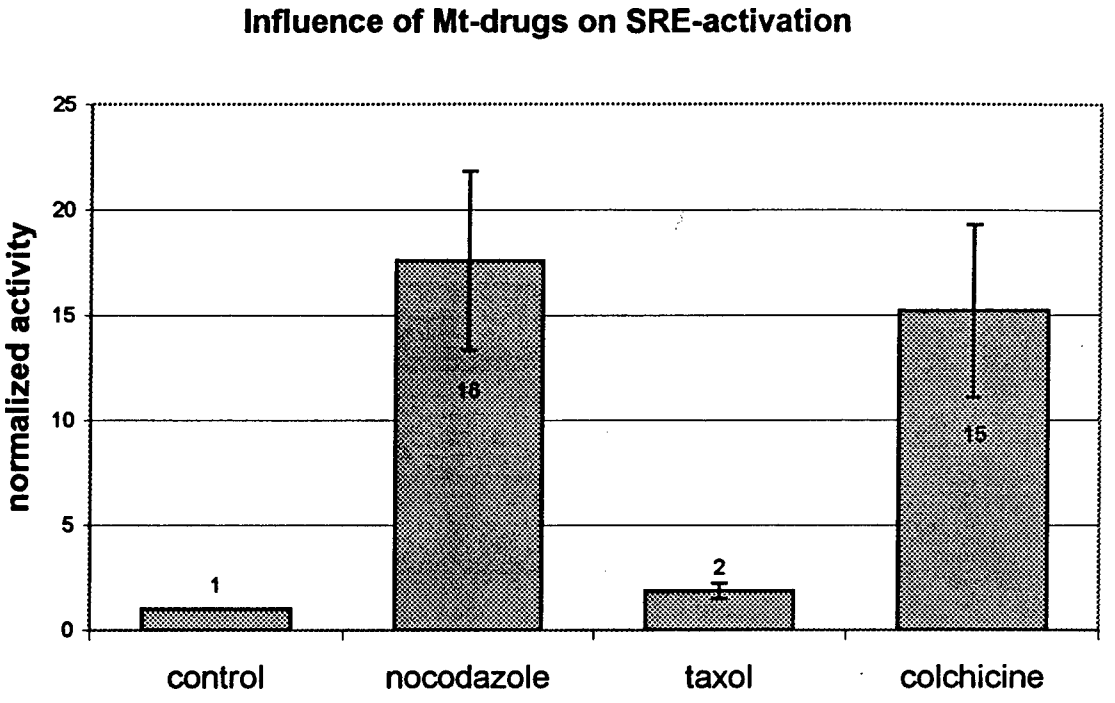


Fig. 8: Nocodazole and colchicine activate SRE luciferase expression. Cos-1 cells were transfected with 0.75 µg of a 2:1 mixture of SRE-luc/pCMV5-lacZ. Microtubule drugs were added 12-14 hrs before lysis at the indicated concentrations (3.3 µM nocodazole, 2.5 µM colchicine, 1 µM taxol). Shown are the mean values +/- standard deviations of at least six independent experiments.

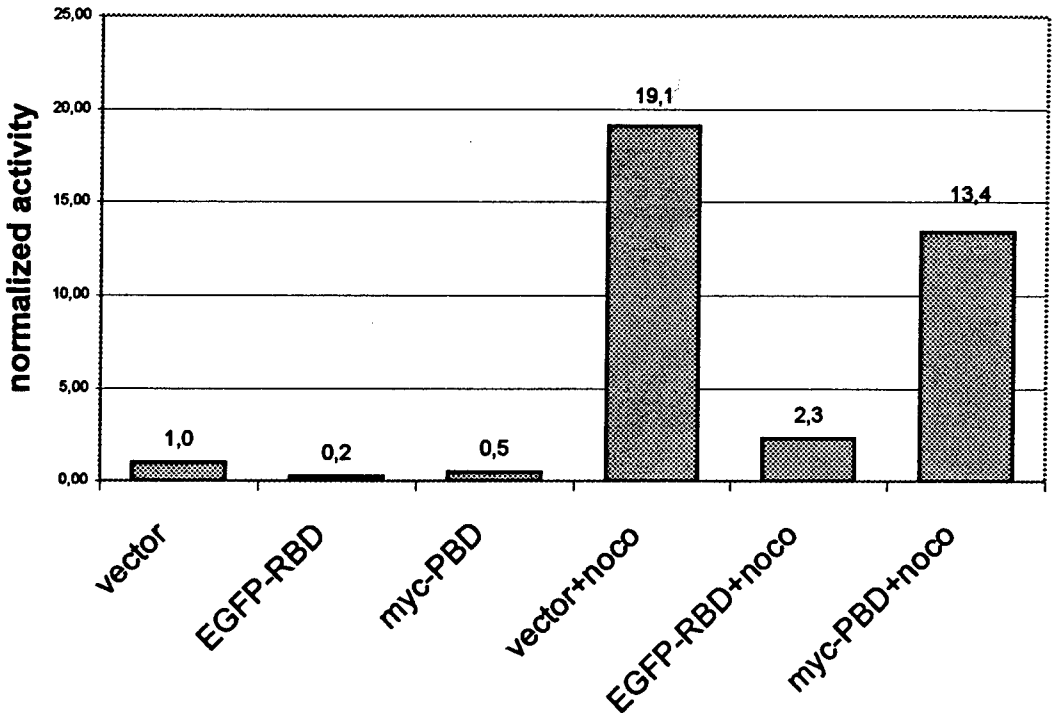


Fig. 9: Nocodazole-induced SRE activation is inhibited by Rho binding domain RBD of Rhotekin. Cos-1 cells were transfected with 0.5 µg of dominant negative constructs, eGFP-RBD fusion or myc-tagged PBD (Rac/Cdc42 binding domain of Pak1), together with 0.75 µg of a 2:1 mixture of SRE-luc/pCMV5-lacZ. Cells were either not stimulated or stimulated for about 14 hrs with 3.3 µM nocodazole (+ noco). A representative experiment is shown.

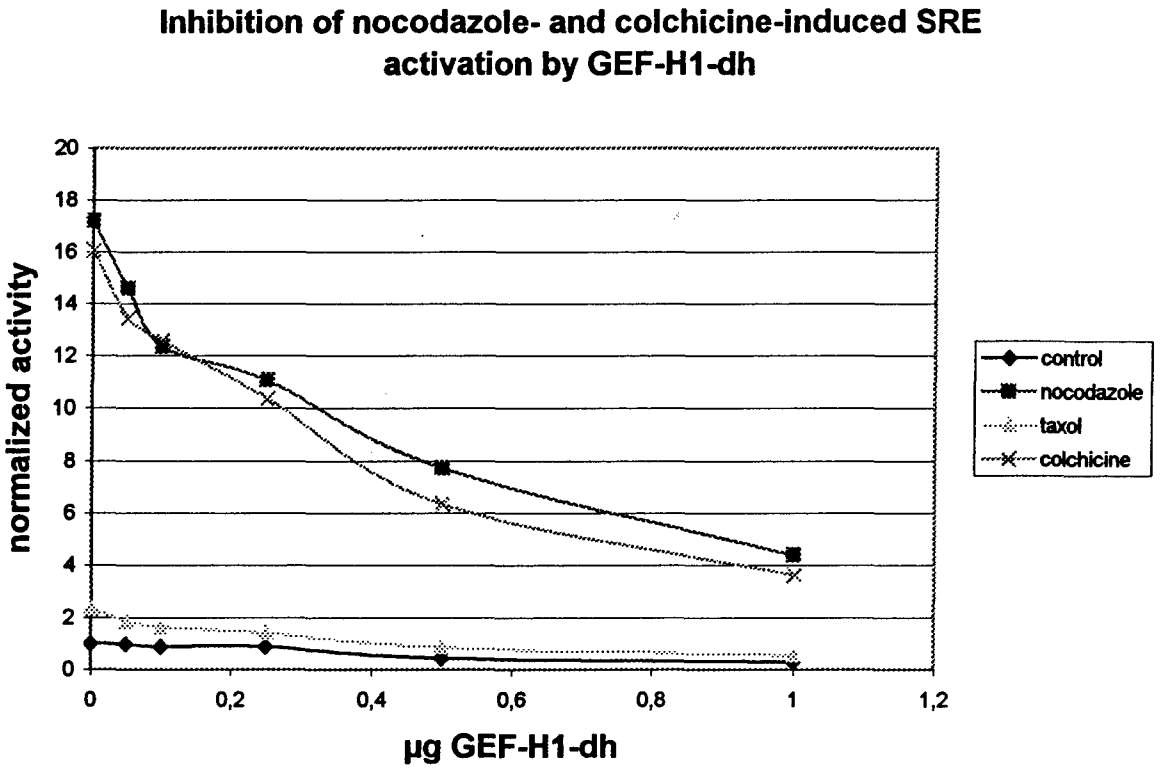


Fig. 10: A dbl-homology domain mutant of GEF-H1 (1-985) inhibits nocodazole- and colchicine-induced SRE-activation in a concentration-dependent manner. Cos-1 cells were transfected with the indicated amounts of GEF-H1-dh and 0.75 µg of a 2:1 mixture of SRE-luc/pCMV5-lacZ. The DNA amount was normalized with pCMV5HA3 vector to 1.75 µg total DNA in each transfection. About 34 hrs post transfection the cells were stimulated with 3.3 µM nocodazole, 1 µM taxol or 2.5 µM colchicine. Cells were lysed about 14 hrs later to determine luciferase and lacZ activity. A representative experiment is shown.

Nucleotide exchange factor GEF-H1 mediates cross-talk between microtubules and the actin cytoskeleton

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ABSTRACT

It is known that regulation of the actin cytoskeleton by microtubules is mediated by Rho family GTPases, but molecular mechanisms linking microtubule dynamics to Rho GTPases have not been identified. We show that the Rho guanine nucleotide exchange factor GEF-H1 is regulated by interaction with microtubules. Microtubule binding-deficient forms of GEF-H1 exhibit higher activity than microtubule–bound forms and induce Rho-dependent changes in cell morphology and actin organization. Microtubule depolymerization induces changes in cell morphology and gene expression similar to those caused by expression of active forms of GEF-H1 and these effects are inhibited by dominant-negative versions of GEF-H1. Thus, GEF-H1 links changes in microtubule integrity to Rho-dependent regulation of the actin cytoskeleton.

INTRODUCTION

Cell migration plays an important role in many physiological processes, including embryonic development, wound healing, and the immune response. Cell migration is powered by the activity of the actin cytoskeleton, with actin polymerization driving leading edge protrusion and acto-myosin contractility promoting cell body advancement. In order to support directional migration, actin dynamics and myosin contractility need to be precisely regulated in a spatially and temporally appropriate manner. Paradoxically, while microtubules do not directly contribute to the generation of forces driving cell migration in most cell types, loss of microtubules prevents directional movement of cells in culture[1], suggesting that microtubules may be involved in regulation of actin-dependent motility. Indeed, fibroblasts lacking microtubules are unable to form lamellipodia in a directional fashion and instead extend new membrane protrusions in a random manner over the cell periphery[1]. The rate of lamellipodial protrusion in these cells is decreased, suggesting that microtubules are necessary to support normal rates of leading edge protrusion[2]. Interestingly, a decrease in lamellipodial activity is also observed in cells treated with low concentrations of the microtubule-depolymerizing drug nocodazole that dampen the dynamics of microtubules without inducing complete depolymerization[3]. Thus, dynamic microtubules appear to be involved in promoting leading edge protrusion. In addition to regulating actin polymerization at the leading edge, microtubules also modulate actin filament organization and myosin contractility in the cell body, since microtubule disassembly promotes formation of stress fibers and enhances contractility[4].

Recent studies have indicated that regulation of the actin cytoskeleton by microtubules relies on the activity of Rho family GTPases (reviewed in[5]). Microtubule growth leads to activation of Rac, which in turn promotes formation of lamellipodia[6], while microtubule disassembly results in activation of Rho, which enhances myosin contractility and stress fiber formation[7-9]. The molecular mechanism through which microtubules modulate activity of Rho GTPases is at present unknown. However, recent identification of guanine nucleotide exchange factors (GEFs) that interact with microtubules[10-12] presents several candidates for the role of a microtubule-regulated Rho/Rac activator. These GEFs, murine Lfc and its human homolog GEF-H1, and p190RhoGEF belong to the Dbl family of Rho activators and contain the characteristic tandem arrangement of a Dbl homology (DH) domain and a pleckstrin homology (PH) domain. At present, there is no data regarding the regulation of the GEF activity of these proteins by microtubules. In the current study, we have analyzed the role of microtubule binding in regulation of the activity of GEF-H1 and provide experimental evidence that GEF-H1 is responsible for regulating Rho activity in response to microtubule depolymerization.

RESULTS

Intracellular localization of GEF-H1

Ren and co-workers characterized the intracellular distribution of the GEF-H1 protein containing amino acids 1-894 and found that it localized to microtubules when expressed in cultured mammalian cells[10]. We cloned full length GEF-H1 cDNA (see Methods) and found that like GEF-H1$^{1-894}$, the full-length GEF-H1 protein also decorated microtubules (Fig. 1). Consistent with the observations of Ren et al.[10], we found that deletion of the entire carboxyl terminus of GEF-H1 resulted in the almost complete loss of microtubule localization (see Fig. 1B for an example of the typical cytosolic localization of the GEF-H1 construct containing amino acids 1-572). The GEF-H1-related KIAA-0651 protein (see Methods) was mostly diffusely distributed and did not localize to microtubules (data not shown). Since KIAA-0651 differs from GEF-H1 primarily in its N-terminal portion, we examined the effect of deletion of the N-terminal segment, including the zinc finger domain, from the GEF-H1 protein. The GEF-H1 construct consisting of amino acids 92-985 did not decorate microtubules (data not shown), indicating that both C- and N-terminal regions of GEF-H1 are important for the interaction with microtubules. Since these observations suggested that the N-terminal Zn finger domain could be involved in interaction with microtubules, we introduced an inactivating point mutation in the Zn finger (C53R) [13]. This mutation led to the loss of microtubule localization (see Fig. 1B).

Expression of EGFP-GEF-H1 or EGFP-GEF-H1$^{1-894}$ proteins in HeLa cells led to appearance of coiled microtubule bundles (Fig. 1B) reminiscent of bundles of stable microtubules induced by overexpression of MAPs (microtubule-associated proteins)[14].

We examined whether overexpression of GEF-H1 led to increased microtubule stability. Bundled microtubules decorated with EGFP-GEF-H1 or EGFP-GEF-H1$^{1-894}$ were more resistant to nocodazole than microtubules in control cells (data not shown). Additionally, microtubules in cells transfected with EGFP-GEF-H1 or EGFP-GEF-H1$^{1-894}$ contained high levels of acetylated α-tubulin, a post-translational modification typically found in stable microtubules[15]. Interestingly, expression of truncated GEF-H1 versions that do not localize to microtubules had no effect on microtubule stability. Since GEF-H1 is a Rho GEF[10] (see also current study) and activation of Rho is known to lead to microtubule stabilization[16], we investigated whether the microtubule-stabilizing effects of GEF-H1 were mediated by a Rho signaling pathway. Inhibition of Rho by the dominant negative RhoA^{T19N}, the Rhotekin Rho-binding domain (RBD), or C3 exoenzyme had no effect on the ability of GEF-H1 to stabilize microtubules. Thus, stabilization of microtubules by GEF-H1 appears to be independent of Rho activation, and may result from the physical association of GEF-H1 with microtubules. In agreement with this hypothesis, a GEF-H1 point mutant deficient in nucleotide exchange activity (GEF-H1^{DHmut}, see below) retained the ability to stabilize microtubules.

Effects of GEF-H1 on cell morphology and actin organization

While investigating intracellular localization of various GEF-H1 constructs, we observed that transfection of HeLa cells with the versions of GEF-H1 deficient in microtubule binding induced dramatic changes in cell morphology (see Fig. 2). HeLa cells expressing EGFP- or HA-tagged GEF-H1$^{1-572}$, GEF-H1$^{92-985}$, KIAA-0651, or GEF-H1^{C53R} acquired an elongated, polarized shape with narrow, finger-like projections along

the cell edge. Mutant GEF-H1 proteins were often enriched in the cell edge projections (Fig. 2B). These projections were distinct in appearance from filopodia, the thin actin-rich membrane protrusions that can be induced in HeLa cells by expression of constitutively active forms of Cdc42. Changes in overall cell morphology induced by mutant GEF-H1 constructs were accompanied by an increase in the number and intensity of actin stress fibers (Fig. 2B). In cells expressing mutant versions of GEF-H1, actin stress fibers often formed well-organized, parallel bundles that extended into the projections of the cell edge. Remarkably, expression of either full-length GEF-H1 or GEF-H1$^{1-894}$ had no noticeable effects on overall cell morphology and organization of the actin cytoskeleton.

To determine whether nucleotide exchange activity of GEF-H1 was required for its effects on cell morphology, we used site-directed mutagenesis to generate a Tyr to Ala amino acid substitution at residue 393 in the conserved QRITKY sequence in the DH (Dbl homology) domain of GEF-H1 (Y393A). As with the analogous mutation in the DH domain of Lbc[17], this substitution completely abolished GEF-H1 nucleotide exchange activity *in vitro* (data not shown). Expression of truncated GEF-H1 constructs (GEF-H1$^{1-572}$ or KIAA-0651) with the Y393A mutation in the DH domain had no effect on cell shape and actin organization (Fig. 2C), indicating that the ability to catalyze nucleotide exchange was crucial for the morphological effects of short versions of GEF-H1. This observation suggested that morphological effects of GEF-H1 constructs were mediated by activation of Rho family GTPases.

In order to identify specific Rho GTPases responsible for the cytoskeletal effects of GEF-H1 expression, we co-transfected HeLa cells with GEF-H1 constructs and the

p21-binding domains of Rhotekin (an effector of Rho) or Pak (an effector of Rac and Cdc42). The Rhotekin RBD and Pak PBD (p21-binding domain) have the ability to bind to active Rho GTPases and specifically inhibit Rho or Rac/Cdc42-dependent pathways, respectively [18,19]. Expression of the Rhotekin RBD blocked GEF-H1 induced alteration of cell morphology and actin organization, while expression of the Pak PBD did not (Fig. 4B). These results suggest that effects of truncated GEF-H1 constructs on cell morphology rely on activation of a Rho-dependent pathway. In agreement with this hypothesis, inhibition of Rho-kinase, a downstream effector of Rho, using the pharmacological inhibitor Y-27632[20] also prevented morphological changes induced by GEF-H1. Additionally, expression of a constitutively active RhoA construct, $RhoA^{Q63L}$, in HeLa cells promoted changes in cell morphology similar to those induced by truncated GEF-H1 constructs.

Nucleotide exchange activity of GEF-H1 *in vitro*

Our analysis of the morphological effects of GEF-H1 in HeLa cells suggested that mutant versions of GEF-H1 modulated cell shape and actin organization via activation of Rho. $GEF\text{-}H1^{1\text{-}894}$ was previously reported to be a GEF for both Rho and Rac[10]. In order to verify that full length GEF-H1 was capable of promoting nucleotide exchange on Rho and to compare nucleotide exchange activity of various GEF-H1 constructs, we performed *in vitro* measurements of nucleotide exchange on RhoA, Rac1, and Cdc42 in the presence of both full-length and truncated versions of GEF-H1. HA- or EGFP-tagged GEF-H1 proteins were immunoprecipitated from Cos-1 cells and used to catalyze exchange of GDP for $[S^{35}]GTP\gamma S$ on Rho GTPases. Both full-length and truncated

versions of GEF-H1 promoted nucleotide exchange on RhoA but not Rac1 or Cdc42 (Fig. 3A). This was in contrast to oncogenic Dbl, which catalyzed nucleotide exchange on all three GTPases. Since only non-microtubule-localized versions of GEF-H1 induced cell shape changes, we hypothesized that deletion of the N- or C-terminal fragments or Zn finger mutation might enhance the activity of GEF-H1 toward RhoA. However, all GEF-H1 constructs tested (GEF-H1, GEF-H1$^{1-894}$, GEF-H1$^{1-572}$, KIAA-0651, and GEF-H1^{C53R}) exhibited similar RhoA guanine nucleotide exchange activity (Fig. 3B and data not shown). Thus, deletion of the N-terminal or C-terminal portions of GEF-H1 or Zn finger mutation did not result in significant changes in *in vitro* guanine nucleotide exchange activity.

Activation of Rho family GTPases by GEF-H1 *in vivo*

To address the apparent discrepancy between the fact that all GEF-H1 constructs have similar activity *in vitro* (Fig. 3) and our observation that only non-microtubule-bound GEF-H1 constructs induced a Rho-dependent change in cell morphology (Fig. 2), we set out to analyze the ability of various GEF-H1 versions to activate Rho GTPases *in vivo*. To this end, we first used RBD/PBD pulldown assays[9,21] to measure the amount of active GTPases present in cells expressing various GEF-H1 constructs. Using these assays, we observed activation of RhoA but not Rac by various GEF-H1 constructs (Fig. 4C and data not shown). While the RBD assay allowed us to measure RhoA activation, we found that variations in the expression of mycRhoA in those experiments that required co-transfection with additional plasmids (e.g., dominant-negative constructs) made routine quantitation difficult. To perform more precise measurements of Rho

GTPase activation, we used a reporter gene assay relying on the ability of Rho to activate transcription of reporter genes fused to the SRE promoter element[22]. Co-expression of GEF-H1 with the SRE-luciferase reporter construct resulted in up-regulation of luciferase expression. This effect was dependent on the nucleotide exchange activity of GEF-H1, as DH domain mutants showed no activity in this assay (Fig. 4D). Interestingly, we observed that non-microtubule-associated GEF-H1 constructs were more active in the SRE reporter assay than the full-length GEF-H1 or GEF-H1$^{1-894}$ (Fig. 4D). Activation of reporter gene expression induced by GEF-H1 was inhibited by Rhotekin RBD but not by Pak PBD (Fig. 4). Thus, activation of SRE by GEF-H1 appears to depend on Rho activity, but not on Rac or Cdc42. These results suggest that the ability of mutant GEF-H1 constructs to induce morphological changes is functionally connected to the higher exchange activity of these proteins revealed by SRE reporter gene activation (and RBD pulldown asay) *in vivo*.

GEF-H1 mediates effects of microtubule depolymerization on Rho activity and cell morphology

Our results indicate that GEF-H1 constructs can be divided into two groups based on their activity in the SRE reporter assay and the ability to promote changes in cell shape and actin organization: the highly guanine nucleotide exchange-active, mutant versions of GEF-H1 and the less active full length and GEF-H1$^{1-894}$ proteins. The highly active GEF-H1 constructs are characterized by the lack of microtubule localization, while less active versions bind to microtubules. Thus, it is reasonable to conclude that microtubule association has an inhibitory effect on GEF-H1 activity and that the loss of

microtubule localization induced by deletion of the N- or C-terminal amino acid sequences of GEF-H1 and Zn finger mutation may represent the cause of higher activity of mutant constructs. If this hypothesis is correct, then disruption of microtubules using microtubule-depolymerizing drugs should lead to activation of endogenous GEF-H1. Indeed, the microtubule-depolymerizing drugs nocodazole and colchicine promoted Rho-dependent activation of SRE-luciferase reporter gene, while the microtubule-stabilizing drug taxol had no effect on SRE reporter gene expression (Fig. 5). Thus, similarly to Rho activation observed upon colchicine treatment of Swiss 3T3 cells[9], disruption of microtubules resulted in activation of an endogenous, Rho-specific regulatory factor in Cos-1 cells.

Treatment of HeLa cells with nocodazole led to changes in cell morphology and actin organization similar to those induced by expression of highly active GEF-H1 constructs. These morphological changes were blocked by expression of RBD (Fig. 5C). These observations, combined with the results of SRE reporter assay, strongly suggest that microtubule depolymerization leads to activation of a Rho-specific nucleotide exchange factor. To verify whether GEF-H1 represents the endogenous factor activated by microtubule disassembly, we tested the ability of the DH domain mutant of GEF-H1 to act as an inhibitor of the SRE activation and morphological changes induced by microtubule depolymerization. We observed that co-expression of GEF-H1^{DHmut} with GEF-H1 partially blocked the effect of GEF-H1 on SRE transcription (data not shown), suggesting that GEF-H1^{DHmut} can act as a dominant-negative inhibitor of GEF-H1 function (see also Methods). Expression of the DH mutant substantially inhibited SRE activation induced by microtubule disassembly but had little effect on SRE activation

induced by Dbl (Fig. 5) or by constitutively active $G\alpha_{12}QL$ or $G\alpha_{13}QL$ that activate Rho through Rho GEFs distinct from GEF-H1[23,24] (not shown). Expression of the DH mutant of GEF-H1 also inhibited nocodazole-induced changes in cell morphology (Fig. 5). Inhibition of nocodazole effects was also seen using a second dominant negative GEF-H1 mutant in which the DH-PH domain tandem had been deleted (data not shown). Wild-type GEF-H1 did not block nocodazole-induced SRE activation and cell shape changes, indicating that the inhibitory effects of GEF-H1^{DHmut} were not due to the stabilization of microtubules by GEF-H1. We therefore conclude that GEF-H1, or a closely related Rho nucleotide exchange factor, is responsible for activation of Rho by microtubule-depolymerizing drugs.

DISCUSSION

In this study we have analyzed the regulation and function of GEF-H1, a microtubule-associated nucleotide exchange factor belonging to the Dbl family of proteins. We observed that deletion of the N- or C-terminal portions of GEF-H1 resulted in the loss of microtubule localization, suggesting that these regions may be involved in interaction with microtubules and/or MAPs. However, isolated N- or C-terminal regions did not localize to microtubules (data not shown), indicating that a combination of protein domains may be necessary for microtubule binding. Previously, Glaven and colleagues observed that the PH domain of Lfc, a mouse homolog of GEF-H1, bound to tubulin[11]. However, we were unable to detect interaction of the isolated PH domain of GEF-H1 with tubulin or microtubules and, since Glaven and colleagues did not test the ability of PH domain to bind to polymerized microtubules, it is unclear whether the PH domain of GEF-H1 contributes to microtubule binding. We found that inactivation of the N-terminal Zn finger domain in GEF-H1 by a single amino acid substitution was sufficient to induce loss of microtubule localization. Thus, the Zn finger domain may play an important role in interaction of GEF-H1 with microtubules.

Expression of GEF-H1 constructs deficient in microtubule binding led to changes in cell morphology including cell retraction and formation of actin stress fibers. These changes are reminiscent of those induced by constitutively active RhoA and suggest that expression of non-microtubule-associated GEF-H1 results in activation of RhoA. GEF-H1$^{1\text{-}894}$ was originally described by Ren et al.[10] as a nucleotide exchange factor for Rho and Rac. However, our data indicate that GEF-H1 can promote nucleotide exchange only

on RhoA, but not Rac or Cdc42 (Fig. 3). Using RBD[9] and PBD[21] pulldown assays to measure the amount of GTP-bound Rho or Rac1, we confirmed that GEF-H1 activated RhoA but not Rac1 in cells expressing GEF-H1 constructs. These data indicate that GEF-H1 is a nucleotide exchange factor for Rho and are in a good agreement with the observation that Lfc, the mouse homolog of GEF-H1, also exhibits specificity for Rho[25]. In support of our conclusions regarding the specificity of GEF-H1 for Rho, our inhibition studies using RBD and PBD indicate that the effects of GEF-H1 on cell morphology and gene expression are mediated by Rho but not Rac or Cdc42.

While all GEF-H1 constructs showed similar guanine nucleotide exchange activity *in vitro*, versions of GEF-H1 deficient in microtubule binding were more active in promoting SRE expression and actin reorganization *in vivo*. Based on this, we conclude that loss of microtubule binding leads to activation of GEF-H1. An alternative explanation is that the N- and C-terminus of GEF-H1 may act together as an autoinhibitory module and that removal of these regions relieves autoinhibition. However, this appears unlikely since the truncated and intact GEF-H1 constructs exhibited equivalent activity *in vitro*. While we were able to observe binding of GEF-H1 to microtubules in detergent-extracted cytoskeletal preparations, we have been unable to reconstitute microtubule binding when microtubules assembled from purified tubulin were added to GEF-H1-containing cell lysates (data not shown). Consequently, we have been unable to demonstrate an inhibitory effect of microtubule binding on GEF-H1 activity *in vitro*. It is possible that reconstitution of GEF-H1/microtubule interactions in vitro requires some additional components or that additional conditions (such as GEF-H1 phosphorylation) need to be met. However, in agreement with our hypothesis that

microtubule binding (either direct or via an associated protein component) downregulates GEF-H1 activity, microtubule depolymerization promoted SRE reporter gene expression and changes in cell morphology identical to those induced by active GEF-H1. These effects of microtubule depolymerization were inhibited by RBD and GEF-H1^{DHmut}. While GEF-H1^{DHmut} was able to act in a dominant-negative fashion in the signaling pathway activated by microtubule depolymerization, it did not inhibit SRE activation mediated by Dbl or by constitutively active $G\alpha_{12}$ and $G\alpha_{13}$ heterotrimeric G-protein subunits that are thought to act through other Rho GEFs such as p115RhoGEF or PDZ RhoGEF[23,24]. Using a dot-blot assay we found that GEF-H1^{DHmut} exhibited only weak binding to RhoA (see Methods), indicating that it was unlikely to exert its dominant-negative effect simply by sequestering Rho. Thus, the dominant-negative effect of GEF-H1^{DHmut} appears to be specific for Rho activation induced by microtubule disassembly and our data strongly indicate that microtubule depolymerization activates a signaling pathway that involves GEF-H1 and Rho.

Regulation of GEF-H1 activity by microtubule association provides a mechanism for modulation of Rho activity in response to changes in microtubule dynamics. Microtubule depolymerization can activate Rho by increasing the amount of free, active GEF-H1 while microtubule assembly downregulates Rho by sequestering and inactivating GEF-H1 (see Fig. 6). This regulatory mechanism may play an important role in processes that rely on coordination of the activities of the actin and microtubular cytoskeletal systems, such as directional cell migration and cytokinesis. In migrating cells, microtubule depolymerization may locally activate Rho in the cell body, resulting in high myosin II activity and thus promoting tail retraction during locomotion. On the

other hand, the prevalence of growing microtubules near the leading edge would result in low Rho activity at the front of the cell, allowing expansion of the leading edge to proceed without being hindered by myosin contractility. Inactivation of GEF-H1 by microtubule polymerization can also provide a molecular basis for the mechanism through which the mitotic spindle defines position of the acto-myosin cleavage furrow. The furrow is always located between the two spindle poles, and one of the models of cytokinesis, the so called "astral inhibition" model, suggests that the presence of growing microtubules near the spindle poles locally inactivates myosin and promotes accumulation of contractile acto-myosin assemblies at the site furthest removed from the microtubule asters[26]. Since cytokinesis relies on the activity of Rho [27,28], it appears very likely that local inhibition of GEF-H1 or a related protein by astral microtubules may be involved in determining the site of Rho activity and myosin contractility in a dividing cell.

In conclusion, we have identified GEF-H1 as a critical biosensor linking cellular actin polymerization and contractility to changes in microtubule dynamics. These data support a regulatory model in which microtubule dynamics actively regulate cellular signaling mechanisms leading to the localized activation of Rho GTPases. The contribution of GEF-H1 to other microtubule-dependent signaling events, and alternate mechanisms for regulation of GEF-H1 activity are currently under investigation.

ACKNOWLEDGEMENTS

The authors are grateful to Yong Ren for providing GEF-H1 1-894 cDNA, to Clare Waterman-Storer and Torsten Wittmann for helpful discussions, to Ben Bohl for help in preparation of recombinant proteins, and to Bruce Fowler for assistance with preparation of GEF-H1 mutants. This work was supported by USPHS grant GM39434 to G.M.B., an Arthritis Foundation Postdoctoral Fellowship to M.K., and Fellowship DAMD17-98-1-8151 from the United States Army Breast Cancer Research Program to F.T.Z.

ABBREVIATIONS

RBD – Rho-binding domain of Rhotekin

PBD – p21-binding domain of Pak1

GEF – guanine nucleotide exchange factor

DH – Dbl homology

PH – pleckstrin homology

MAP – microtubule-associated protein

METHODS

DNA constructs

A plasmid containing a cDNA encoding the 894 amino acid long GEF-H1 protein was kindly provided by Yong Ren[10]. Our re-sequencing of this plasmid revealed several discrepancies with the originally published sequence. Correction of the sequencing errors led to a frame shift that removed the stop codon present in the ORF of the original GEF-H1 sequence. The corrected ORF encoded a protein of 985 amino acids. Comparison of the full-length GEF-H1 sequence with other protein sequences in the database revealed 88 % overall sequence identity with the 985 amino acid long mouse protein Lfc[11] (accession number 9957220). This high degree of sequence similarity indicated that Lfc represented the mouse homolog of human GEF-H1. Additionally, database search uncovered a protein that likely represents an alternatively spliced form of GEF-H1. This protein is encoded by a human cDNA clone KIAA-0651[29] and differs from GEF-H1 only in its N-terminus, which does not contain a zinc finger motif (Fig. 1A). We obtained a cDNA clone encoding KIAA-0651 (accession number AB014551) from the Kazusa DNA Research Institute. All GEF-H1 and KIAA-0651 constructs were subcloned into pCMV5-HA_3 (triple hemagglutinine epitope) or pCMV5-EGFP vectors.

GEF-$H1^{DHmut}$ was prepared by site-directed mutagenesis to generate a Tyr to Ala mutation at residue 393 in the conserved QRITKY sequence in the DH domain of GEF-H1. We examined the ability of GEF-$H1^{DHmut}$ to bind RhoA using a dot-blot assay. GST-tagged DH-PH domain protein containing the inactivating DH mutation and recombinant RhoGDI (used as a positive control) were spotted on nitrocellulose and

overlayed with RhoA(GDP). Bound RhoA was detected after washing using a rabbit polyclonal Rho antibody, followed by I^{125} Protein A. The DH mutant exhibited only weak RhoA binding that was typically an order of magnitude less than the amount of RhoA bound to RhoGDI on the same blot, over an equivalent range of protein concentrations.

The p21-binding domain of Pak1 PBD (amino acids 67-150) was fused to the myc-epitope at the amino terminus and inserted into pCMV6.

Cell transfection and microscopic observation

For microscopic observations, HeLa cells were grown on glass coverslips in 35 mm dishes in DMEM containing 10% fetal calf serum and transfected using 0.75 μg of each DNA construct and 5 μl of LipofectAmine reagent (Gibco BRL) following the manufacturer's instructions. 24 hrs post-transfection, cells were either examined using phase-contrast microscopy combined with fluorescence microscopy to detect EGFP fluorescence or fixed and processed for immunofluorescence staining using anti-HA antibodies (UBI, Covance), anti-tubulin antibody (DM1A, Sigma), or Alexa-phalloidin (Molecular Probes). Fixation, immunofluorescence staining, and imaging were performed as described[30]. For some experiments, cells were incubated with 10 μM nocodazole for 1-2 hrs or 10 μM Y-27632 for 30-60 min.

***In vitro* exchange assays**

Hemagglutinin- or EGFP-tagged GEF-H1 versions were expressed in Cos-1 cells and immunoprecipitated using anti-HA or anti-GFP monoclonal antibodies and Protein

G-sepharose. A protocol using immunoprecipitates for exchange assays was used essentially as described[31] except that [^{35}S]GTPγS was used instead of [^{32}P]GTP. All nucleotide exchange reactions were performed for 15 minutes at 30°C.

RBD and PBD pulldown assays

HeLa cells were co-transfected with GEF-H1 constructs and myc-tagged RhoA or Rac1 and lysed after 24 hrs of expression. RBD and PBD assays were performed as described[9,21].

SRE reporter gene assay

For transient transfection of Cos-1 cells we used 0.5 μg pSRE-luciferase and 0.25 μg pCMV5/lacZ per 35 mm dish as indicator and transfection control plasmids, respectively. GEF-H1 constructs and other plasmids were added at 0.25 μg per well, vector DNA was added to normalize the total DNA amounts. Cells were transfected using Lipofectamine according to the manufacturer suggestions except that the serum amount was kept at 0.5 % throughout the transfection period. Cell lysates were prepared 48 hrs post transfection. Luciferase and galactosidase activity were measured using the Luciferase Assay Kit (Promega) and GalactoLight Kit (Tropix). Microtubule drugs were added 12-14 hrs before lysis at the indicated concentrations (3.3 μM nocodazole, 2.5 μM colchicine, 1 μM taxol).

FIGURE LEGENDS

Figure 1. Panel A. A schematic drawing showing the domain structure of the full length GEF-H1 (amino acids 1-985) and various expression constructs used in this study with the positions of the first and the last amino acid indicated. The protein encoded by the KIAA-0651 cDNA clone is identical to GEF-H1 along most of its length (solid line) but differs from GEF-H1 in the N-terminus (dashed line).

Panel B. Intracellular localization of GEF-H1 constructs. HeLa cells transfected with EGFP-tagged GEF-H1 constructs were fixed and stained with anti-tubulin antibody. Full length GEF-H1 and GEF-H1$^{1-894}$ co-localized with microtubules. GEF-H1$^{1-572}$ and GEF-H1^{C53R} (Zn finger mutant) exhibited mostly diffuse cytoplasmic localization with some enrichment at the tips of the projections of the cell edge. Scale bars – 20 μm.

Figure 2. Changes in cell morphology and actin organization induced by the expression of GEF-H1 constructs.

Panel A. HeLa cells transfected with EGFP-GEF-H1 constructs were imaged using phase-contrast microscopy. Cells expressing GEF-H1 constructs (asterisks) were identified by EGFP fluorescence. Cells expressing full length GEF-H1 and GEF-H1$^{1-894}$ were similar in their morphology to non-transfected cells while cells expressing GEF-H1$^{1-572}$ and GEF-H1^{C53R} had an elongated, polarized shape and formed numerous projections along the edge.

Panel B. Transfected HeLa cells were fixed and stained with fluorescent phalloidin to label actin and with antibodies against HA tag. Cells expressing GEF-H1$^{1-572}$ and GEF-

$H1^{C53R}$ exhibited more intense actin stress fiber labeling than non-transfected cells or cells transfected with full length GEF-H1 and GEF-$H1^{1-894}$. Stress fibers in GEF-$H1^{1-572}$ and GEF-$H1^{C53R}$ expressing cells formed bundles extending into the projections of the cell edge.

Panel C. HeLa cells expressing HA-GEF-$H1^{1-572}$ with an inactivating mutation (Y393A) in the Dbl homology domain (DH mutant) were fixed and stained as in panel B. Cells expressing GEF-$H1^{1-572,DHmut}$ were similar in their actin organization to non-transfected cells. All scale bars – 20 μm.

Figure 3. *In vitro* guanine nucleotide exchange activity of GEF-H1.

Panel A. Oncogenic Dbl or full length GEF-H1 immunoprecipitated from transfected Cos-1 cells were used to catalyze binding of [S^{35}]GTPγS to Rac1, Cdc42, or RhoA as described in Methods. To promote forced nucleotide loading (EDTA/Mg), EDTA was added to 13.3 μM and subsequently nucleotide binding was stabilized by addition of $MgCl_2$ to 30 mM. Background level of nucleotide binding observed in the absence of GEFs or EDTA was substracted from all the values, and the level of nucleotide binding in the presence of EDTA was set to 100%. While EDTA and Dbl promoted nucleotide exchange on all three GTPases used, GEF-H1 catalyzed nucleotide exchange only on RhoA.

Panel B. HA-tagged GEF-H1 constructs were immunoprecipitated from Cos-1 cells and used to catalyze nucleotide exchange on RhoA (right panel). All constructs were present in equivalent amounts in the immunoprecipitates (left panel) and showed similar

exchange activity. Results shown in A and B are representative of at least 2 independent experiments.

Figure 4. Activation of Rho by GEF-H1 constructs *in vivo*.

Panel A. Cos-1 cells were transfected with reporter plasmids, GEF-H1$^{1-572}$, and RBD or PBD. Reporter gene expression was measured as described in Methods. The amount of luciferase expressed was divided by the amount of expressed β-galactosidase to adjust for variations in transfection efficiency. SRE expression induced by GEF-H1$^{1-572}$ was inhibited by coexpression of RBD. Results shown are representative of at least 2 independent experiments.

Panel B. HeLa cells were transfected with HA-GEF-H1$^{1-572}$ and EGFP-RBD or EGFP-GEF-H1$^{1-572}$ and myc-PBD. Expression of RBD prevented induction of morphological changes by GEF-H1$^{1-572}$ while expression of PBD had no effect. Bar – 20 μm.

Panel C. HeLa cells co-transfected with GEF-H1 constructs and myc-RhoA were lysed and GTP-bound RhoA was precipitated using GST-RBD. The amount of RhoA bound to RBD was analyzed by Western blotting with anti-myc antibody. Cells expressing GEF-H1$^{1-572}$ or KIAA-0651 contained more GTP-RhoA than cells transfected with full length GEF-H1.

Panel D. SRE-luciferase expression induced by various GEF-H1 constructs was measured as described for panel A. Each of the values shown represents the mean +/- standard deviation of at least 2 independent experiments. GEF-H1$^{1-572}$, KIAA-0651, and GEF-H1^{C53R} were more active in promoting SRE-luciferase expression than full length

GEF-H1 and GEF-H1$^{1-894}$, while GEF-H1 constructs with inactivating mutation (Y393A) in the Dbl domain (GEF-H1^{DHmut}) showed no activity.

Figure 5. Effect of microtubule depolymerization on SRE activation and cell shape.

Panel A. Cos-1 cells were transfected with reporter plasmids, treated with microtubule drugs and processed for measurements of luciferase expression as described in Methods. Microtubule depolymerization using nocodazole (3.3 μM) or colchicine (2.5 μM) promoted SRE activation while microtubule stabilization with taxol (1 μM) had no effect. Shown are the mean +/- standard deviations of six different experiments.

Panel B. Cos-1 cells transfected with RBD or PBD and reporter plasmids were treated with 3.3 μM nocodazole and used to measure luciferase activity. Expression of RBD blocked nocodazole-induced SRE activation.

Panel C. HeLa cells transfected with EGFP-tagged constructs were imaged using phase-contrast and fluorescence microscopy. EGFP-expressing cells are indicated by asterisks. Treatment with 10 μM nocodazole resulted in formation of numerous projections along cell edge while cells expressing RBD and GEF-H1^{DHmut} maintained circular shape characteristic of non-treated cells. Expression of EGFP alone did not block nocodazole-induced cell shape changes. Scale bar – 20 μm.

Panel D. Top. Cos-1 cells transfected with reporter plasmids and varying amounts of GEF-H1^{DHmut} DNA were treated with microtubule drugs and luciferase activity was measured as described for panel A. Expression of GEF-H1^{DHmut} inhibited SRE activation by microtubule-depolymerizing drugs. Results shown are representative of at least two independent experiments.

Bottom. Comparison of inhibitory effects of GEF-H1^{DHmut} on SRE activation by oncogenic Dbl and nocodazole. Each data point represents the mean +/- standard deviation of 3 independent experiments.

Figure 6. A model for regulation of GEF-H1 activity by microtubules. GEF-H1 is inactive when bound to microtubules and becomes activated when microtubules are depolymerized, either as a result of inherent instability or after treatment with microtubule-depolymerizing drugs. Activated GEF-H1 promotes binding of GTP to Rho, leading to Rho activation, which in turn results in up-regulation of myosin II contractility, stress fiber assembly, and SRE-regulated gene expression.

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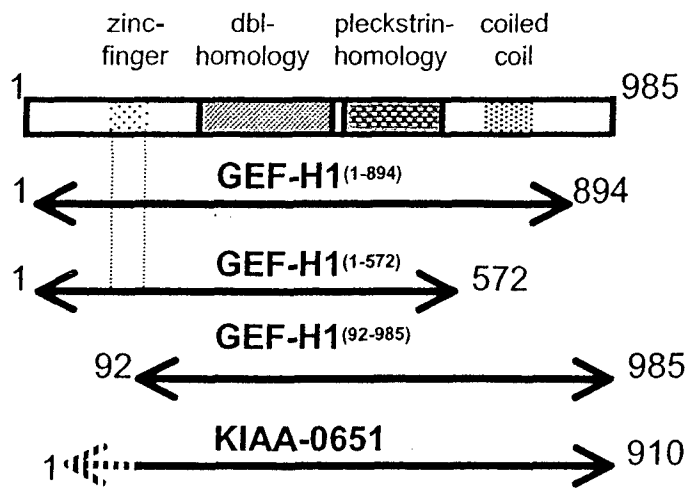
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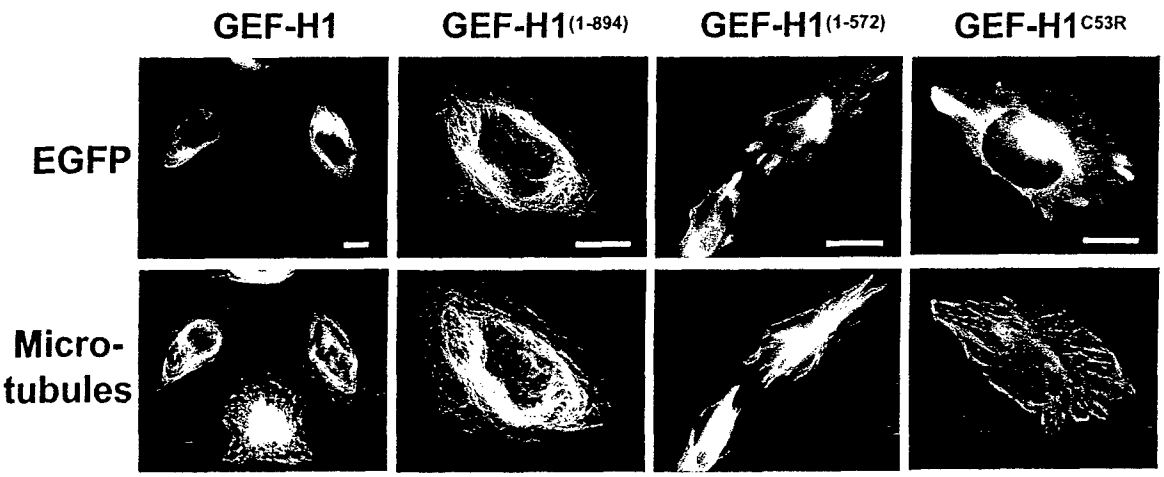
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Figure 1

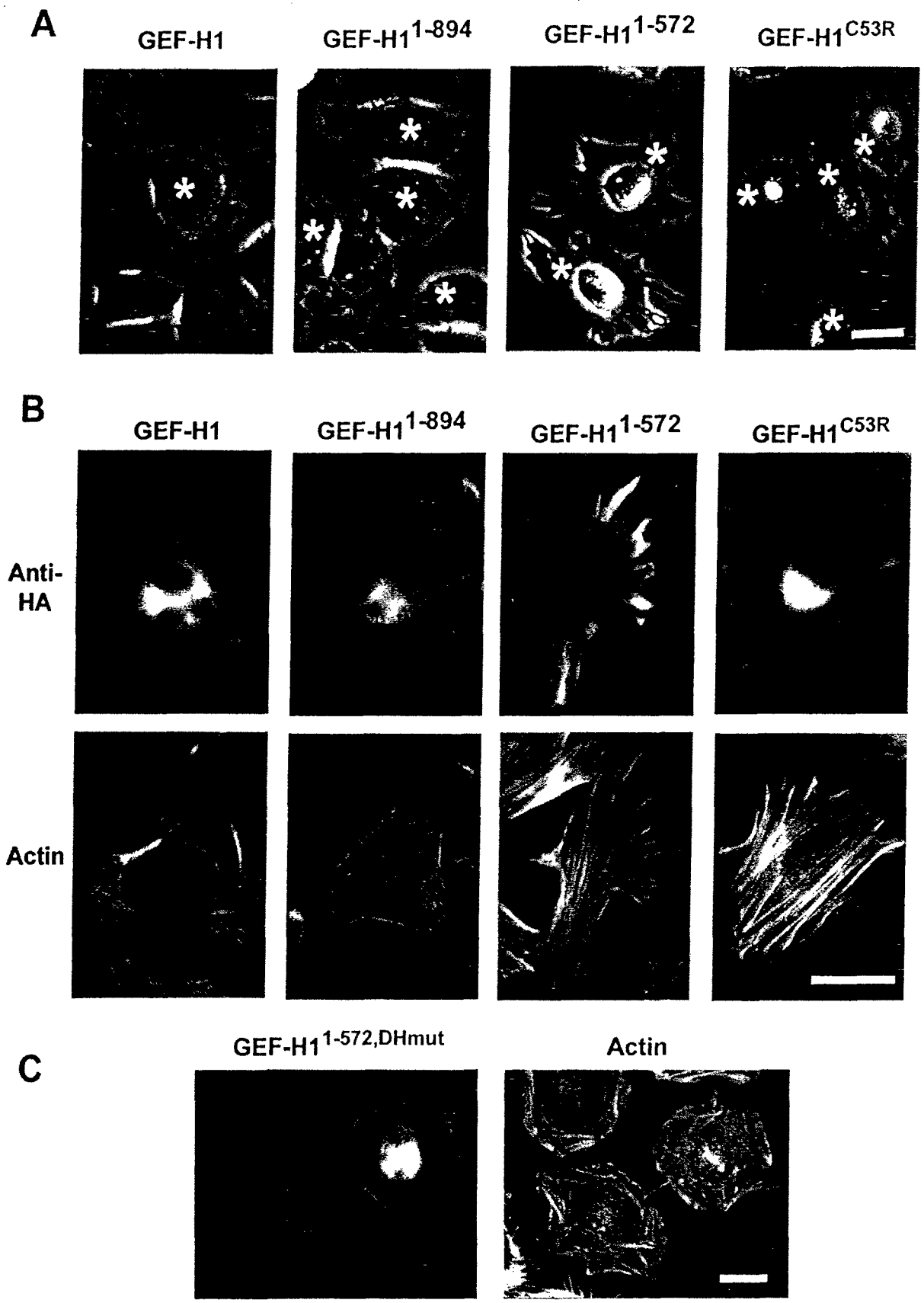
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Figure 2

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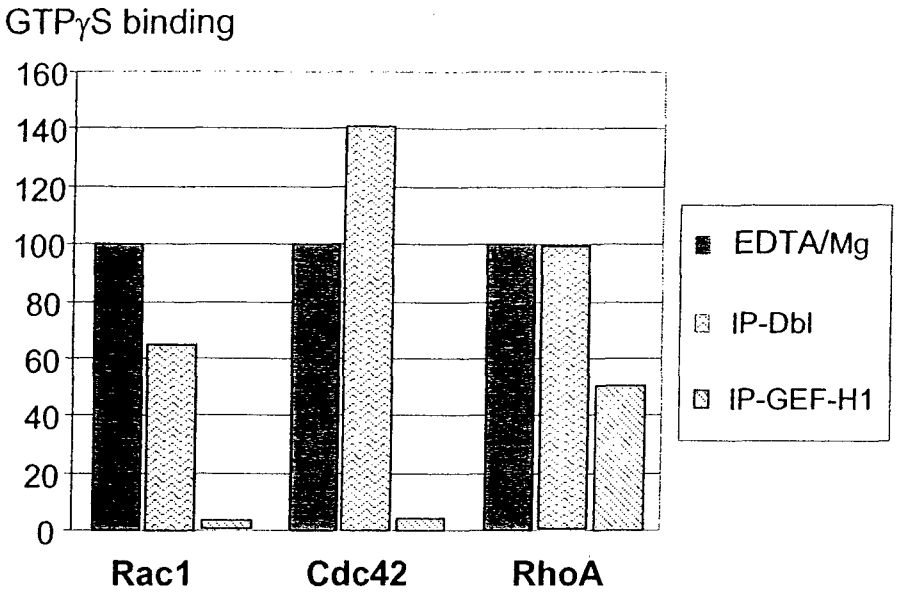
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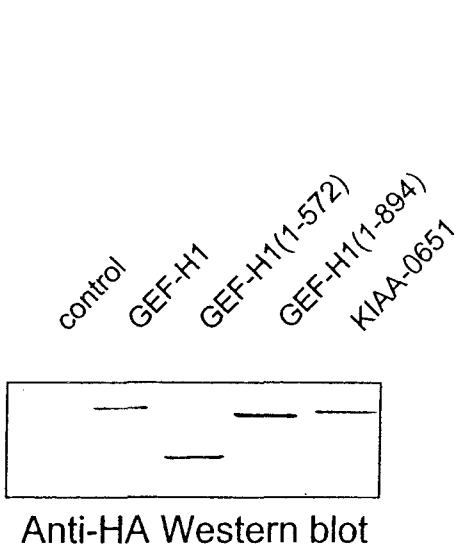
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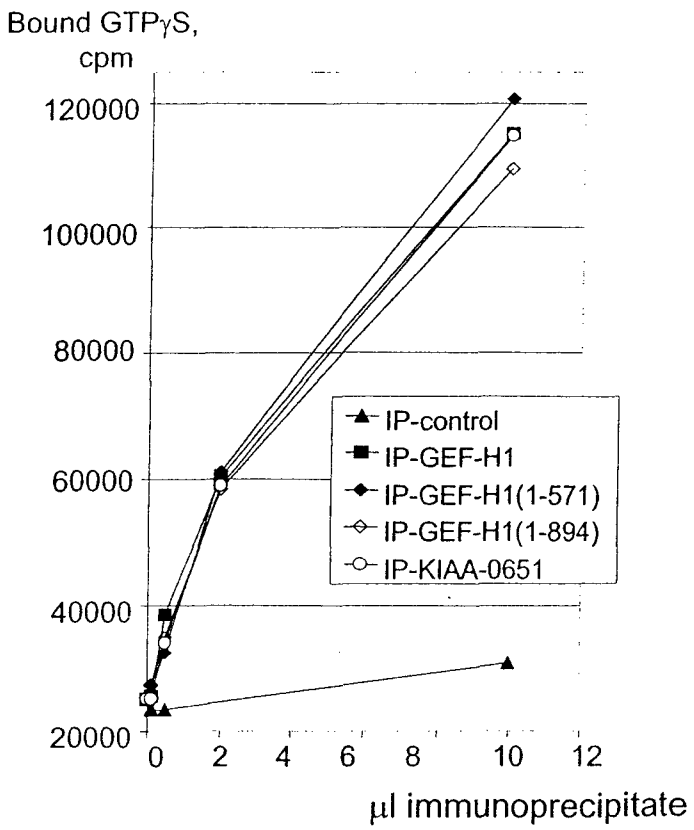
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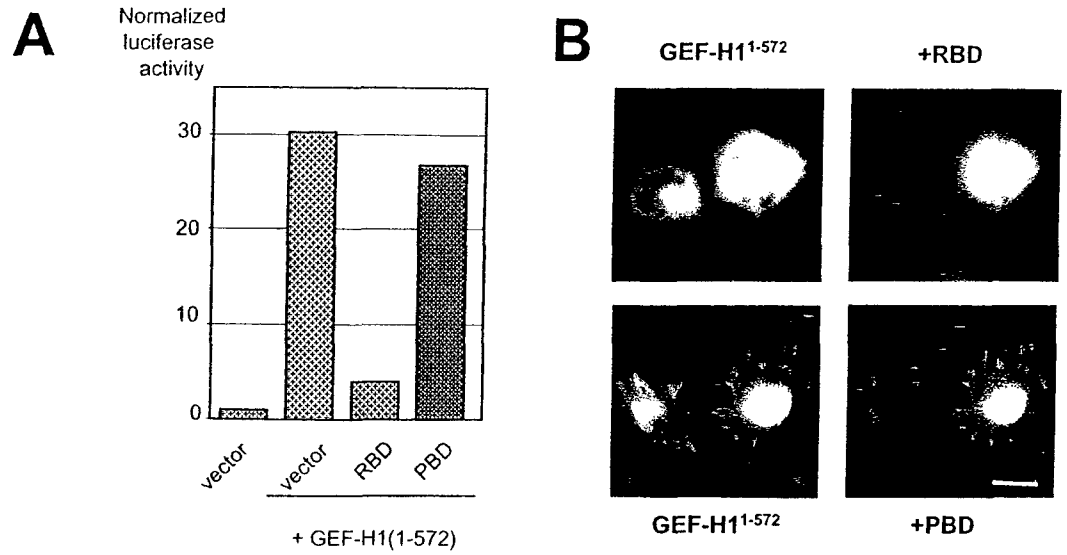
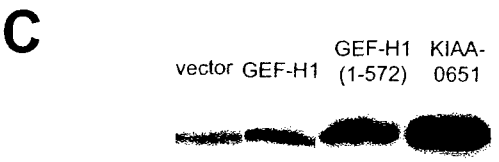
Figure 3


A
Normalized luciferase activity
30
20
10
0
vector
vector
RBD
PBD
+ GEF-H1(1-572)
B
GEF-H1 1-572
+RBD
GEF-H1 1-572
+PBD


C
vector
GEF-H1
GEF-H1 (1-572)
KIAA-0651

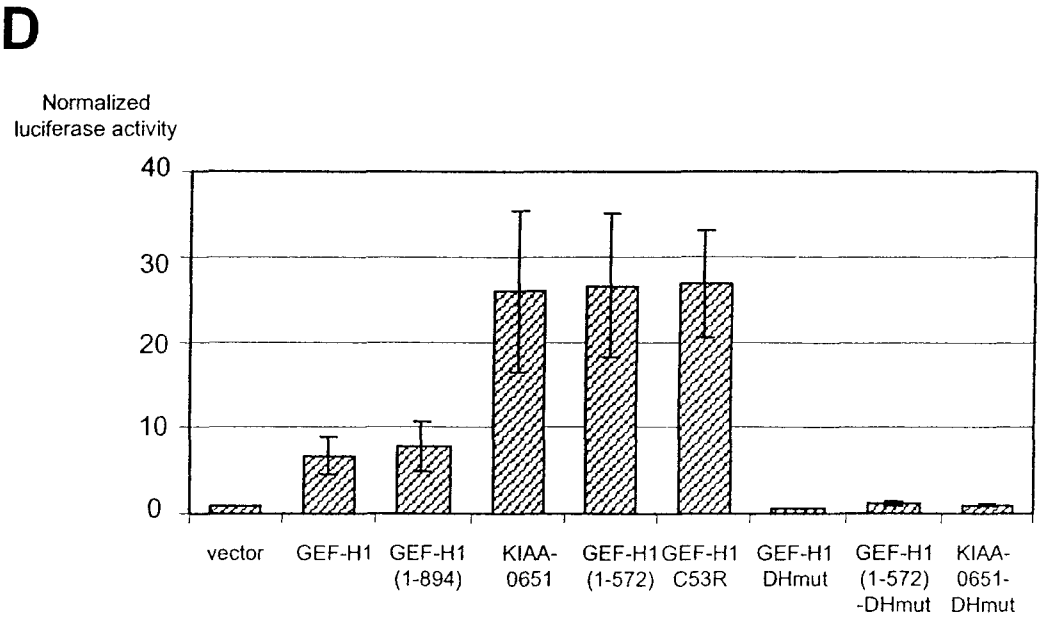

D
Normalized luciferase activity
40
30
20
10
0
vector
GEF-H1
GEF-H1 (1-894)
KIAA-0651
GEF-H1 (1-572)
GEF-H1 C53R
GEF-H1 DHmut
GEF-H1 (1-572) -DHmut
KIAA-0651-DHmut

Figure 4

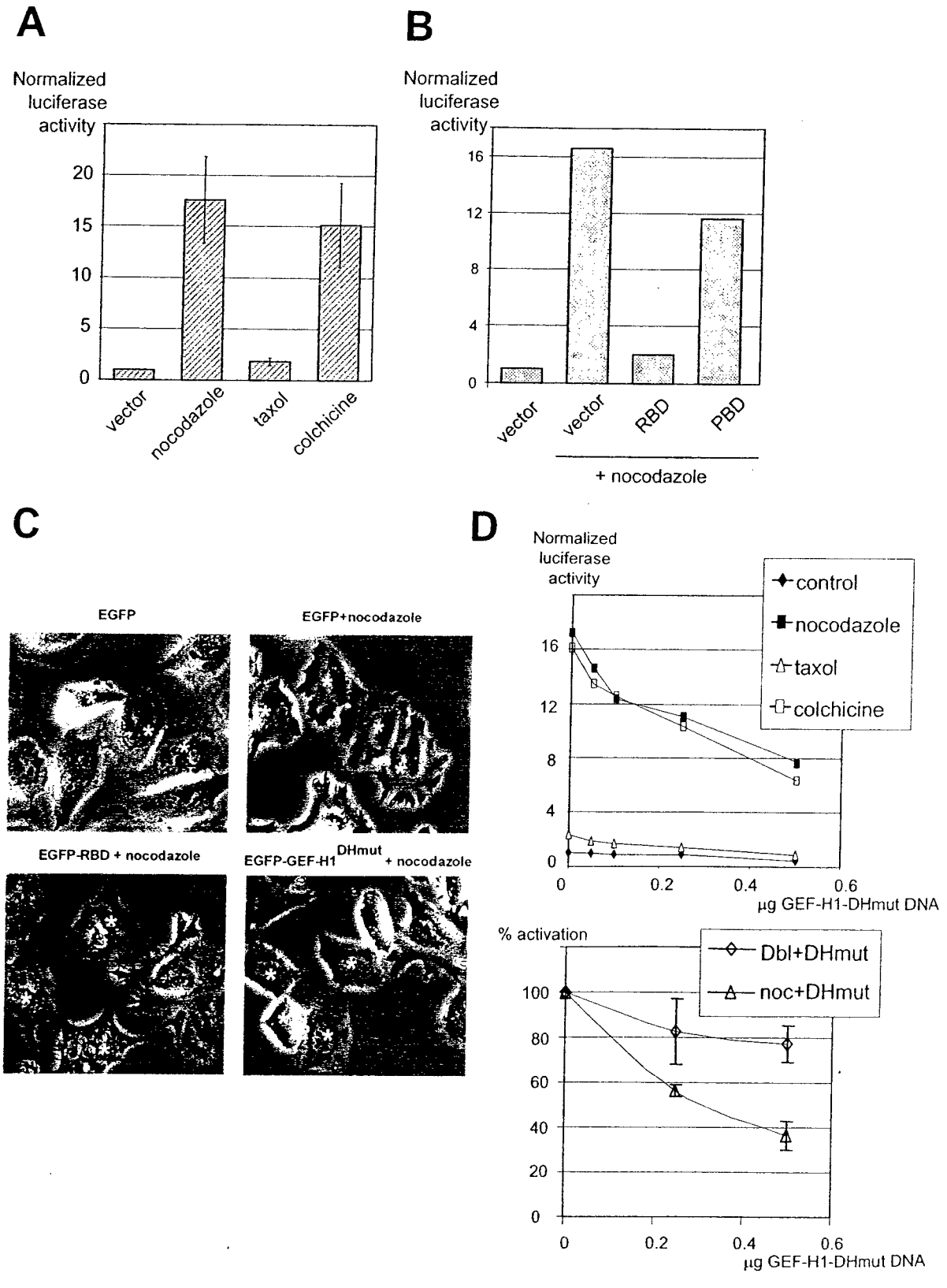

A
Normalized luciferase activity
20
15
10
5
0
vector
nocodazole
taxol
colchicine
B
Normalized luciferase activity
16
12
8
4
0
vector
vector
RBD
PBD
+ nocodazole
C
EGFP
EGFP+nocodazole
EGFP-RBD + nocodazole
EGFP-GEF-H1DHmut + nocodazole
D
Normalized luciferase activity
control
nocodazole
taxol
colchicine
16
12
8
4
0
0
0.2
0.4
0.6
µg GEF-H1-DHmut DNA
% activation
Dbl+DHmut
noc+DHmut
100
80
60
40
20
0
0
0.2
0.4
0.6
µg GEF-H1-DHmut DNA

Figure 5

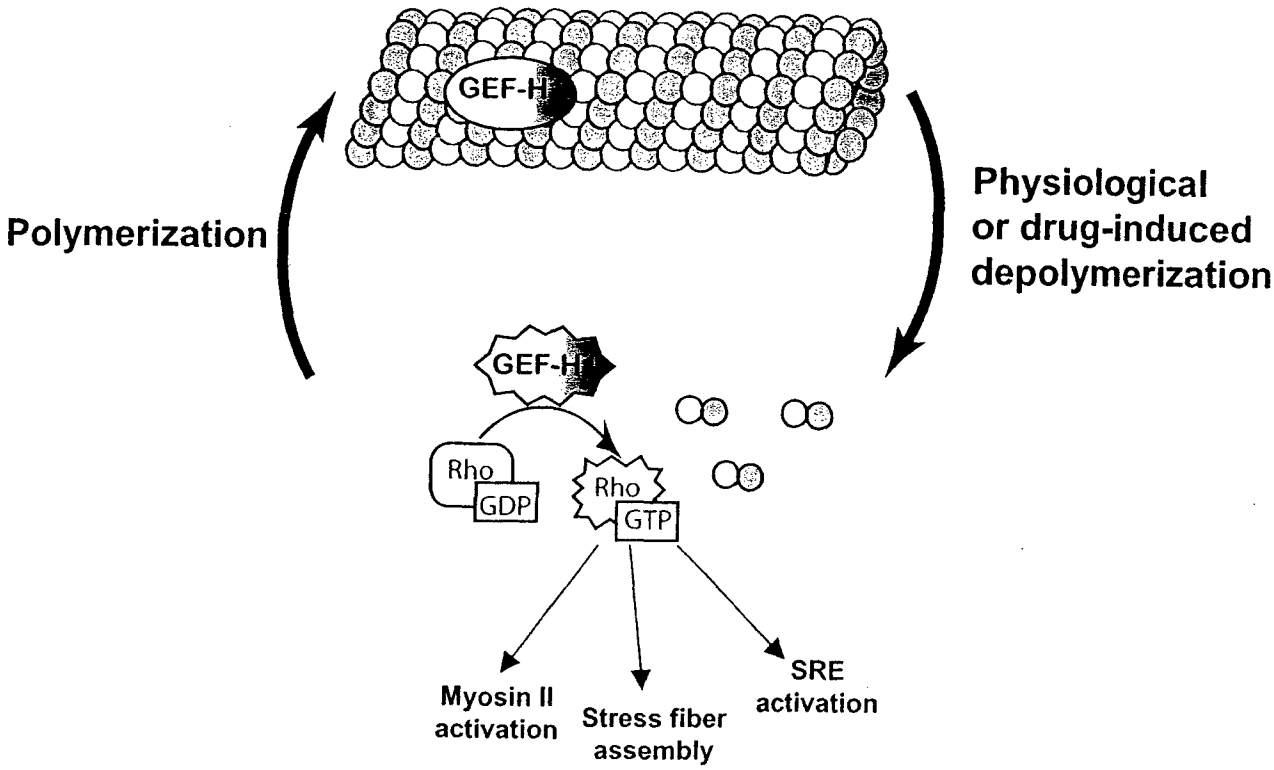

GEF-H1
Polymerization
Physiological
or drug-induced
depolymerization
GEF-H1
Rho
GDP
Rho
GTP
Myosin II
activation
Stress fiber
assembly
SRE
activation

Figure 6

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 21 Feb 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLLIS M. RINEHART
Deputy Chief of Staff for
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